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STUDIES ON THE CARDIAC STOMACH OF A STARFISH, PATIRIA MINIATA (BRANDT)[1]

JOHN MAXWELL ANDERSON

Department of Zoology, Cornell University, Ithaca, N. Y.

Patiria miniata, the cushion star or sea-bat of the Pacific Coast of North America, is perhaps best known to zoologists as a ready source of materials for the study of asteroid embryology. Of equal interest, however, are its voracious appetite and its unusual feeding habits. *Patiria* functions as an omnivorous scavenger of both plant and animal materials and to some degree at least as a predator on sessile gastropods. Unlike *Asterias* and *Pisaster*, it does not open bivalves; but it has developed to a much greater extent than any of the free-armed starfishes the ability to evert the cardiac stomach through the mouth and to employ it as a feeding organ of great effectiveness. Almost every individual observed undisturbed in a tide pool is found with its remarkably voluminous cardiac stomach fully everted and widely spread. Specimens maintained in an aquarium spend a good share of the time with their everted stomachs closely applied to the glass, perhaps, as suggested by MacGinitie and MacGinitie (1949, p. 227), digesting accumulated growths of diatoms. Bits of kelp or other seaweed, cracked snails, large pieces of mussel flesh, living limpets, or even smaller specimens of *Patiria* are covered and held against the substratum by the body of the starfish, then enveloped by the spreading folds of the everted stomach (Fig. 4) until completely digested. Even small pieces of soft food, which the animal is capable of ingesting, are first covered and wrapped in folds of the stomach before being brought into the cavity of the gut.

Such observations suggest that the cardiac stomach of *Patiria* functions in a highly specialized manner, being more frequently and more extensively exposed to the external environment than the stomachs of other familiar starfishes. It seemed likely that the functional peculiarities of this organ might be reflected in significant structural specializations, probably involving regional differentiation of the stomach wall and a particularly well-developed system of fibrous attachments for holding and retracting the everted stomach. Although *Patiria* is a relatively common intertidal species in much of its range along the Pacific coast, no detailed anatomical or histological studies of its digestive tract appear to have been made. The present

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study was undertaken to provide a basis for comparison in a contemplated series of experiments, and as a contribution to knowledge of comparative and functional anatomy and histology in the digestive systems of asteroids. The results of this study have brought to light several interesting features of the cardiac stomach of *Patiria* that have apparently not been described previously, either for this species or for any of its relatives.

It is a pleasure to express to the Director and Staff of the Hopkins Marine Station my appreciation for their hospitality and helpful cooperation during the conduct of these investigations.

Material and Methods

Small to moderate-sized specimens of *Patiria miniata* were collected in tide pools near Pacific Grove, California, and maintained in the laboratory in running sea-water. Many observations of normal feeding behavior were made on these animals, which were periodically provided with a variety of food such as cracked snails, opened mussels, kelp fronds, living limpets, etc.

For anatomical studies, animals were immersed in isotonic $MgCl_2$ (8% in tap water) until flaccid, then opened by cutting through the body wall all around the margin of the body and removing the aboral part, transecting the digestive tract at the junction between cardiac and pyloric stomachs. The cardiac stomach and its retractor harness were then studied in their normal positions; or, by cutting all the strands binding the stomach to the ambulacral ossicles and transecting the esophagus, the stomach could be removed from the body. Portions of such excised stomachs, maintained in a relaxed state by continued immersion in $MgCl_2$ solution, were spread and pinned out with fine glass needles in small wax-bottomed dissecting trays. After completion of studies of these spread segments in the living condition, the salt solution was decanted and the preparation flooded with Helly's fluid or Zenker-acetic. Fixation was continued for 24 hours; the fixed tissue was then removed from the wax, washed for several hours in running water, trimmed, dehydrated, and imbedded in paraffin. Carefully oriented serial sections were cut at 7 μ, in a plane parallel to the mouth. These "horizontal" sections were carried through a variety of staining routines, for various purposes. For general histological observations, Harris' hematoxylin followed by eosin or light green was used; for delineation of cell boundaries, cytoplasmic granules, and muscular and connective-tissue fibers, Mallory's phosphotungstic acid hematoxylin (PTA) proved excellent. Glycogen and other polysaccharide complexes were demonstrated by the use of a periodic acid-Schiff routine (Lillie, 1954, p. 123), controlled by salivary digestion. Metachromatic substances were revealed by overnight staining in dilute toluidine blue, followed by alcoholic dehydration and differentiation. A standard Feulgen technic was used to identify nuclei by selective staining of their DNA content.

Certain special experimental procedures will be described later, in connection with the observations they were designed to elucidate.

Observations

The retracted cardiac stomach occupies a large part of the central cavity of the disk. It lies in the form of a more or less regular series of pouches, extending from

the main lumen both perradially and interradially, above and between the proximal ambulacral ossicles of the rays. These pouches are separated by medially and upwardly directed folds that extended almost to meet a series of similar folds hanging from the roof of the pyloric stomach. A relatively slight constriction divides the stomach into the conventionally recognized cardiac and pyloric portions, leaving a comparatively broad passageway between them; it is into this passageway that the medial folds of the cardiac stomach protrude. The constriction separating the upper and lower portions of the stomach is girdled by a glistening band of connective-tissue fibers related to the retractor harness of the cardiac stomach.

The retractor mechanism

What may be termed the extrinsic elements of the retractor harness consist in each ray of a pair of fan-shaped bundles extending from broad origins along the sides of the ambulacral ridge to rather more restricted attachments on the wall of the cardiac stomach (Fig. 2). These bundles bear a superficial resemblance to the extrinsic strands described for *Asterias forbesi* by Anderson (1954) but differ markedly in detail. Whereas in *Asterias* the extrinsic components consist of thick marginal bands supporting thinner, mesentery-like sheets, those of *Patiria* are thick and tough throughout, and each of the fan-shaped bands comprises four strands. The shortest and most proximal of these originates on the connective-tissue coat lateral to the first or proximal ambulacral ossicle and follows a short course aborally, skirting between the perradial and interradial pouches, to insert on the fibrous girdle described above. The longest of the extrinsic strands forms the upper border of the fan-shaped band, originating lateral to the ambulacral ridge in the region of the tenth to twelfth ossicle and inserting also on the fibrous girdle of the stomach, adjacent to the attachment of the shortest strand. Between the shortest and the longest lie two intermediate strands, somewhat thicker, originating alongside the ambulacral ridge, passing between the perradial and interradial pouches, and disappearing proximally under the perradial pouch. Here these strands appear to end rather abruptly but actually break up into branching and rebranching intrinsic retractor fibers spreading widely over the surface of the cardiac stomach as they approach the oral end of this organ. It will be noted that the stomach of *Patiria* lacks the nodules characteristic of the retractor system of *Asterias*, the conspicuous fibrous knots upon which all extrinsic strands insert and from which all intrinsic strands branch. Instead, in *Patiria* the stomach is provided with the fibrous girdle which is related only to those elements of the extrinsic system that do not contribute to the branching intrinsic fiber systems.

The intrinsic fiber patterns on the wall of the cardiac stomach in *Patiria* generally resemble those of *Asterias*. In the portion of the stomach pertaining to each of the rays, two major bands pass from the blunt ends of the intermediate extrinsic strands, bound together initially but immediately diverging widely to course diagonally downward. These soon become closely applied to the outside of the stomach and pass underneath the visceral peritoneum to establish contact with the muscular and connective-tissue layers of the stomach wall. Each of the major strands now branches into two, and below the points of divergence of the four strands thus produced a series of four additional dichotomies occurs. Eventually, the final products of all these binary divisions, tapering slowly and growing progressively smaller as they approach the lower end of the stomach, disappear

as fine fibers blending into the stomach wall at a line near the upper limit of the esophagus. The paired extrinsic bands in each of the rays are thus continued as intrinsic strands intimately connected with the fabric of the stomach wall and spreading to 64 widely dispersed terminal attachments at its lower end; in a 5-rayed specimen, the intrinsic retractor fibers are distributed to a total of 320 ultimate points of attachment, all arranged along a regular line (*cf.* Fig. 1). For purposes of identification in subsequent discussions and to avoid confusion with other components to be described later, the branching strands spreading from the ends of the extrinsic bands will be referred to as *Class 1* fibers.

Histological examination shows that these intrinsic strands, like those of *Asterias*, are composed of varying mixtures of connective-tissue and muscle fibers, bound together and enclosed by the typical cuboidal epithelium of the visceral peritoneum (Fig. 7). As the strands branch, becoming smaller and more intimately connected with the stomach wall, they continually contribute both connective-tissue and muscle fibers to the corresponding layers in the wall of the stomach (Fig. 8). In their lowest levels, near the esophagus, the small terminal branches can be recognized only in sections, appearing as characteristically staining, somewhat thickened groups of connective-tissue fibers running longitudinally beneath the peritoneum. The larger Class 1 fibers are attached to the stomach wall only along their sides, often leaving a long, tubular cavity beneath the strand. In such spaces, the outer surface of the stomach appears to be composed of its muscular and connective-tissue coats, as the retractor strands run inside the peritoneum (Figs. 5, 6, 17).

The Class 1 fibers, in addition to making direct contributions to the muscular and connective-tissue layers of the stomach, are also closely related to a secondary system of strands to be designated as *Class 2* fibers. These are found in a zone lying roughly between the second and fifth forks of the Class 1 system. They consist of very large numbers of tiny, parallel strands which spring at closely spaced but irregular intervals from the stomach wall, in close proximity to the courses of Class 1 fibers (Fig. 6). The Class 2 strands run a horizontal course, leaving the stomach wall to branch perhaps once before re-entering the wall and disappearing again, in the unsupported pouches between the Class 1 branches (Fig. 3). The horizontal fibers are longest high in the stomach, where they arise near the widely separated major branches of the Class 1 system; they grow progressively shorter in lower levels, where the Class 1 branches approach each other more closely. The small and inconspicuous Class 2 fibers are of particular interest in that they constitute a previously undescribed feature of the retractor mechanism and one that does not exist in *Asterias*.

Study of the Class 2 fibers in histological sections shows that they originate in the muscle layers of the wall of the stomach and hence are formed almost exclusively of strands that can be identified as muscle fibers (Fig. 6). These join and pass out through the more superficial connective-tissue layer to form cylindrical bundles of parallel fibers, each enclosed by a layer of peritoneal cells (Fig. 9). After a longer or shorter course through the perivisceral coelom near the outer surface of the stomach, the strands re-enter the wall, penetrate the connective-tissue layer again, and contribute their fibers in a spreading pattern to the muscle layers of the stomach (Fig. 10). Class 2 bundles in their free courses over the surface of the stomach range approximately from 9 to 14 μ in diameter.

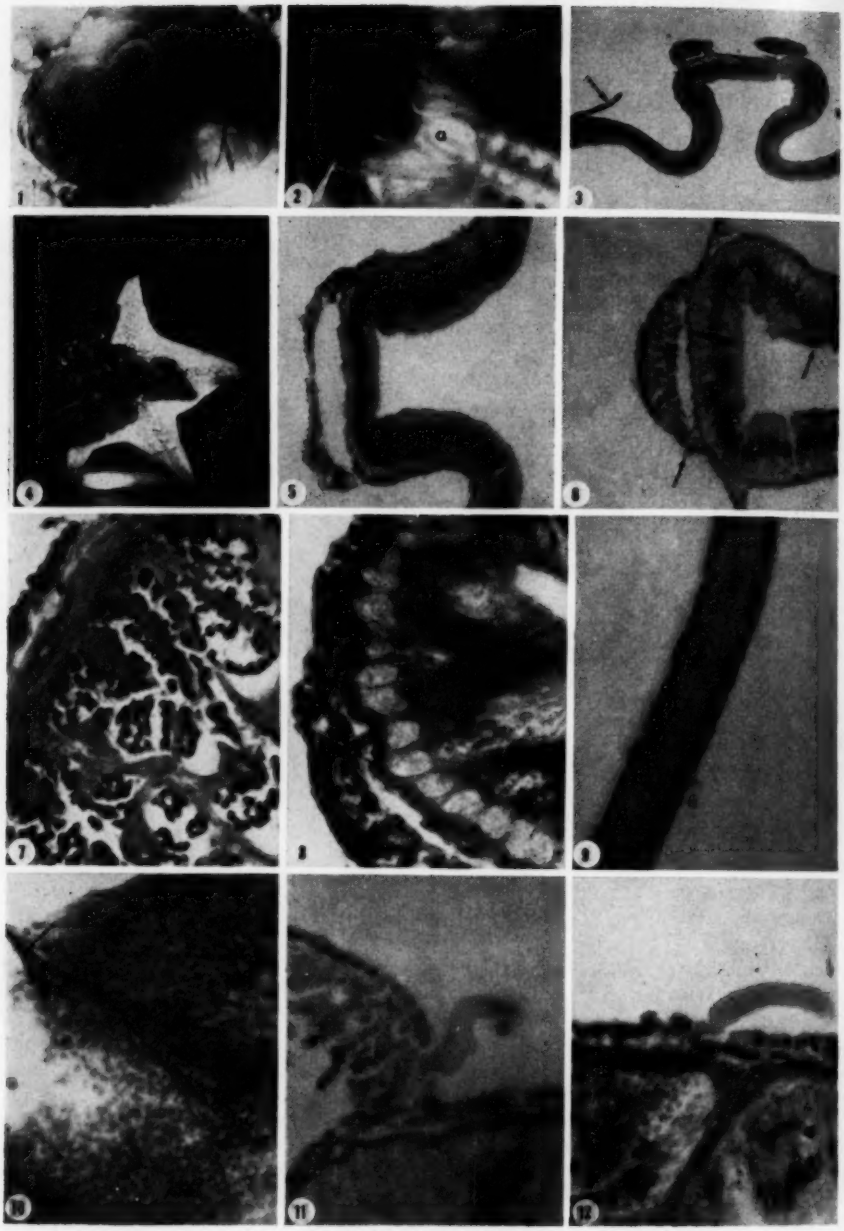
Still another group of fibers is found outside the stomach of *Patiria,* lying in parallel longitudinal array about its lower end. These, to be referred to as *Class 3* fibers, are the most slender of all the retractor and restraining bands of the stomach wall. Each appears to consist of a single, hyaline, homogeneous strand of extracellular fibrous material enclosed by the vastly extended cytoplasm of the cell that produced it; the nucleus of the cell lies in a thickened area of the cytoplasm somewhere along the length of the strand (Fig. 13). Class 3 fibers exist in a considerable size range, from just under 2 μ to more than 8 μ in diameter. The coarsest are also the longest, originating in the connective-tissue components of small Class 1 fibers, passing out through the peritoneum into the coelom, turning downward toward the peristome, and finally penetrating the peritoneum again to contribute several branching processes to the connective-tissue layer of the esophagus or peristome. The origin of such a coarse fiber from a small Class 1 strand is shown in Figure 11; Figure 12 shows the insertion of a similar strand in the peristomial region. The most numerous of the Class 3 fibers are smaller and shorter, having both ends anchored in the connective-tissue layer of the stomach itself, not related to other strands. These run brief courses across folds in the stomach wall (Fig. 13) or across the deep bay formed by the reflection of the esophagus above the horizontal peristomial membrane. It should be emphasized that all of the Class 3 fibers, of whatever size, consist of single, nucleated cells enclosing fibrous extracellular strands; they contain no muscle fibers, and they are never clothed by a peritoneal layer as fibers of Classes 1 and 2 always are. Like the Class 2 fibers, these constitute a new feature, previously undescribed.

In a restricted zone at the lowest end of the stomach, fibers of all three classes are present; that is, Class 1 fibers give off coarse Class 3 fibers in the zone near the esophagus in which short Class 2 strands also occur. Here, of course, the horizontal Class 2 fibers and the longitudinal Class 3 strands run perpendicular to each other. By far the majority of the Class 3 fibers, however, are found below the level occupied by the lowest of the Class 2 strands.

The stomach wall

In *Patiria,* as in *Asterias,* the spreading branches of the intrinsic fibers (Class 1) are accompanied by underlying specializations in the structure of the stomach wall. These consist of branching gutters, beginning at the lower end of the stomach beneath the terminal branches of the Class 1 fibers and running upward, converging and joining with each other to form progressively larger channels that ultimately widen and fade out at approximately the level where the primary strands of the intrinsic retractor system become attached to the stomach. The regular, consistent, branching patterns of these gutters are conspicuous in vesicles of the everted cardiac stomach; Figure 1 shows such a pattern in a single everted vesicle, and in Figure 4 they are clearly visible in several parts of the widely spread stomach.

Horizontal sections near the oral end of the stomach reveal the intimate relationship that exists between the gutters and various categories of intrinsic fibers. Class 1 fibers are attached longitudinally along the evaginated folds that form the gutters, and the horizontal strands of the Class 2 fibers extend from the shoulders of these folds to attach again in unspecialized areas between neighboring grooves. This arrangement of the Class 2 strands makes it possible for them to maintain the gutters by puckering the wall of the stomach and creating the inwardly-directed



EXPLANATION OF FIGURES.

Figures 1 and 2 are photographs of living preparations, and Figure 4 is a photograph of a living animal. The remaining figures on this and the following plate are photomicrographs of tissues fixed in Helly's fluid (except Figs. 7 and 20, fixed in Zenker-acetic), sectioned at 7μ, and stained as indicated. The magnifications given are approximately correct for the figures as they appear here, after enlargement and reduction.

FIGURE 1. Portion of the everted cardiac stomach, showing a part of the system of gutters in the stomach wall associated with Class 1 retractor fibers. Note the regular line along which the smallest branches begin, in the region termed the esophagus, and the convergence of the gutters towards the mouth (arrow). 4 ×.

FIGURE 2. Elements of the extrinsic retractor system in one ray, after removal of the roof of the animal with all aboral parts of the digestive tract. Note the connective-tissue girdle surrounding the stomach (arrow); just below this point the shortest and longest retractor strands of one side insert on the girdle, close together. Within the angle they form lie the two intermediate strands which pass onto the surface of the stomach (to left) and become continuous with Class 1 intrinsic fibers. The proximal ambulacral ossicle is indicated at *a;* this is partially covered by a perradial pouch extending from the stomach between the two short retractor strands. 4 ×.

FIGURE 3. Horizontal section passing through the level of confluence of two small gutters, low in the stomach; the Class 1 fibers associated with the gutters have not joined at this level. The arrow indicates a horizontal Class 2 fiber passing into the plane of the section and inserting on the stomach wall; its origin on the shoulder of the ridge nearby is not shown; *cf.* Figure 6. Phosphotungstic acid (PTA) hematoxylin; 70 ×.

FIGURE 4. A large individual feeding on an algal frond; the vesicles of the broadly-everted cardiac stomach have passed through holes in the frond and spread out against the glass wall of the aquarium. The arrow indicates a typical vesicle; note the great size of the stomach, and the conspicuous gutter-and fiber patterns in its vesicles. Approximately 0.25 ×. (This photograph was made by Dr. Allahverdi Farmanfarmaian of the Hopkins Marine Station and is printed with his permission.)

FIGURE 5. Section similar to Figure 3, higher in the stomach, with two small Class 1 fibers joining. Note the flat floor of the gutter and the general differences in the epithelium as between the floor and the sides of the gutter. PTA hematoxylin; 145 ×.

FIGURE 6. Horizontal section, still higher in the stomach. This section demonstrates the relationship between the three classes of intrinsic retractor fibers: a large Class 1 fiber is shown in cross-section, broadly attached by its margins to the ridge above the gutter. A pair of Class 2 fibers proceeds laterally (top and bottom, in the figure) from the shoulders of the ridge, and a coarse Class 3 fiber is shown at its origin from one side of the Class 1 strand (left arrow). Note the close relationship between the fibers of Class 1 and Class 2. In the side-wall area indicated by the right-hand arrow, note the distal deposits of basophilic granules in the epithelial cells; these are always lacking in cells lining the floor of the gutter. The dark bodies in the floor are mucous goblets. Harris' hematoxylin, light green; 145 ×.

FIGURE 7. Portion of a cross-section of a major Class 1 fiber, high in the stomach. At upper left, the peritoneal covering, followed by a heavy connective-tissue coat; the darkly-staining masses are shrunken bundles of muscle fibers, bound together and separated from each other by layers of connective tissue. Zenker-acetic fixation, PTA hematoxylin; 650 ×.

FIGURE 8. Floor of a gutter, with attachment of a Class 1 fiber; the arrow indicates a large connective-tissue bundle passing into the connective-tissue layer of the stomach wall from the retractor strand (lower left). Above this, the muscular components of the intrinsic fiber mingle with the muscular layers of the stomach wall. Lumen of the stomach to right, perivisceral coelom to left. Note the thick nerve layer, represented by the light-colored areas in arcades between the bases of the epithelial cells. PTA hematoxylin; 650 ×.

FIGURE 9. Longitudinal section of a Class 2 fiber, composed of parallel muscle fibers enclosed by peritoneum. Harris' hematoxylin, light green; 650 ×.

FIGURE 10. Tangential section of stomach wall, showing the insertion of a Class 2 fiber (arrow) and the wide distribution of its branching strands in the muscle layer of the stomach. Note that these muscle fibers are not arranged in circular and longitudinal layers but run in all directions. PTA hematoxylin; 320 ×.

FIGURE 11. The origin of a coarse Class 3 fiber from the attachment-point of a Class 1 fiber to the stomach wall. Note the clear, homogeneous nature of the Class 3 fiber and the shriveled coat of cytoplasm surrounding it. This fiber, turning downward towards the mouth, passes out of the section at the blurred point, where it runs alongside a second, smaller Class 3 fiber shown here in cross-section. Harris' hematoxylin, eosin; 650 ×.

FIGURE 12. The insertion of a coarse Class 3 fiber by branching processes penetrating the peritoneum and muscle coats to enter the connective-tissue sheet of the stomach wall. The very heavy connective-tissue layer with inward extensions is characteristic of the peristomial region, where the longest of the Class 3 fibers terminate. PTA hematoxylin; 650 ×.

PLATE II

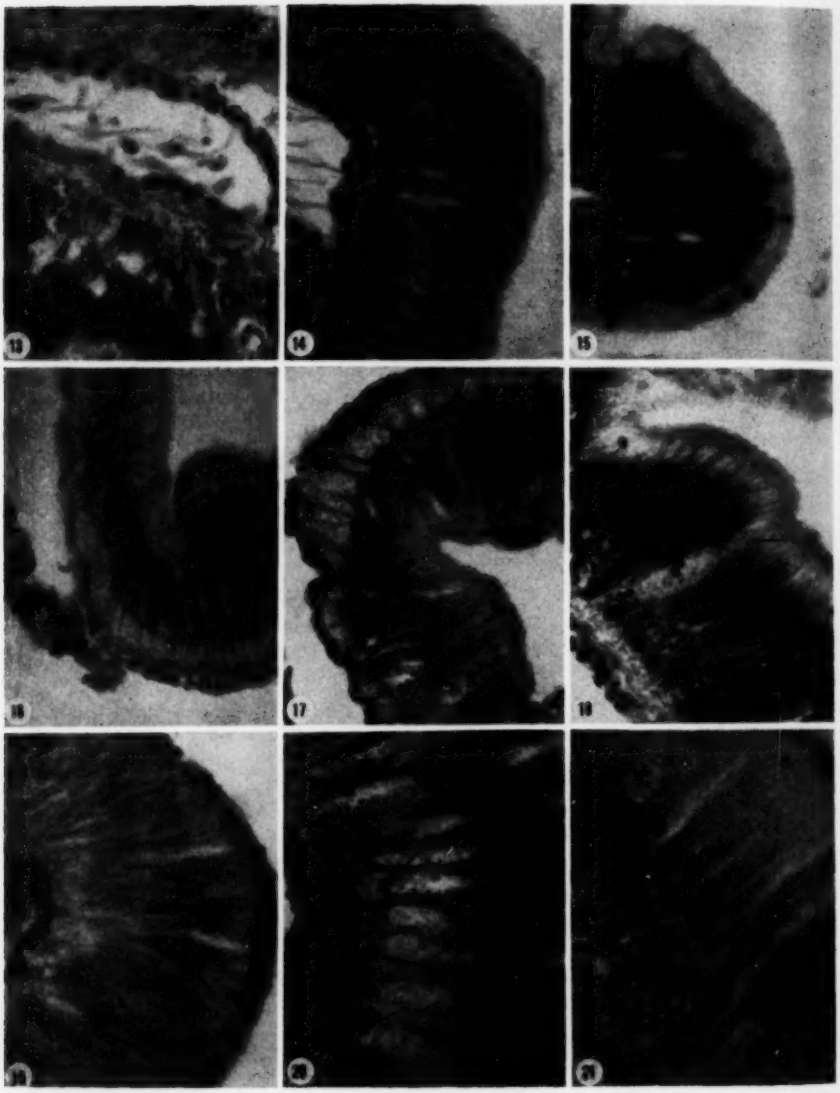


FIGURE 13. A group of short Class 3 fibers lying in the coelom in a fold of the stomach wall. Note nuclei in the cytoplasmic masses attached to some. PTA hematoxylin; 650 ×.

FIGURE 14. Nature of the stomach wall at the lip of a gutter, in the lower part of the stomach. Note the tall epithelial cells with long, dense nuclei, characteristic of these regions

folds forming their sides. It should be noted that the preparation of this material for sectioning involved stretching and spreading segments of the stomach and pinning them to flat wax plates; even such treatment does not obliterate the gutters, which must be considered a permanent feature of the wall of the stomach.

The general histology of the stomach is typical of that repeatedly described, with minor variations, for the wall of the asteroid digestive tract (Hamann, 1885; Cuénot, 1887; Ludwig and Hamann, 1899; Chadwick, 1923; Hayashi, 1935; Anderson, 1954). A flagellated cuboidal epithelium clothes the outer surface. Just inside this lie two layers of muscle fibers, which Hamann considers as representing an outer longitudinal and an inner circular layer; in *Patiria,* muscle fibers run in all directions, forming an irregular network (Fig. 10), and it is difficult to determine which of them make up the principal circular and longitudinal strands. Inside the muscle-fiber network a connective-tissue layer is encountered. This varies in thickness, being most conspicuous in the neighborhood of Class 1 strands, which contribute fibers to it (Fig. 8). The connective-tissue layer serves also as a basement membrane, to which are attached the proximal ends of the very tall, slender, columnar epithelial cells that line the stomach and constitute the principal layer of its wall. In many areas the basal portions of these epithelial cells join

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as of the side walls of the gutters. Lumen of stomach to right, coelom to left; the random fibers lying in the coelom are small Class 3 strands. PTA hematoxylin; 650 ×.

FIGURE 15. Similar epithelium lower in the stomach, near the esophagus, showing a few secretory cells packed with coarse spherules. These are interpreted as "mulberry cells." Note the distal row of flagellary basal granules and the brush border. PTA hematoxylin; 650 ×.

FIGURE 16. Portion of the wall of a small gutter, at the zone of transition between low cells with rounded nuclei in the floor (above) and taller cells with elongate nuclei in the wall (right). Lumen of stomach at upper right, coelom to left. The irregular strand passing upward at the left is part of the Class 1 fiber associated with this gutter; note that the peritoneal layer mounts over the outside of this strand and is absent from the stomach wall beneath it. *Cf.* Figures 5, 6, and 17. PTA hematoxylin; 650 ×.

FIGURE 17. Similar section through the floor of a small gutter; lumen of stomach to right, coelom to left, with an attachment point of the associated Class 1 fiber just above left center. Note the thick nerve layer in the epithelial arcades, the small spherical nuclei high in the epithelium (amoebocyte nuclei?), and the absence of a peritoneal layer beneath the Class 1 strand. Several cysts are apparent above the nerve layer in the floor of the gutter. PTA hematoxylin; 450 ×.

FIGURE 18. Transition zone in the side of a small gutter. Note the characteristic elongated nuclei, distal differentiations of cells, a large mucous goblet (arrow) with shrunken nucleus, and numerous cysts lying above the basal nerve layer. Lumen of stomach to right, floor of gutter at top. PTA hematoxylin; 650 ×.

FIGURE 19. Typical epithelium near the lip of a gutter, showing localizations of PAS-positive material after salivary digestion. Note the conspicuous connective-tissue layer (left) and granular deposits in the distal ends of the epithelial cells. Lumen of stomach to right. Periodic-acid-Schiff, after salivary digestion, counterstained with Weigert's acid iron-chloride hematoxylin and light green; 650 ×.

FIGURE 20. Cysts between the bases of tall epithelial cells. These contain clear, refractile, homogeneous bodies; the nuclei (arrow) are the only Feulgen-positive elements associated with the cysts. Zenker-acetic, PTA hematoxylin; 650 ×.

FIGURE 21. Typical rounded-nucleus epithelium spreading from shallow gutters high in the stomach. Peritoneum at lower left, lumen of stomach at upper right. Note the long, clear mucous goblets, and the numerous cysts; compare the appearance of the contents of these cysts with those in Figures 8, 17, 18, 20. PTA hematoxylin; 650 ×.

together in bundles before inserting on the basement membrane, forming arcades of various sizes occupied by the fibers of the nerve layer (Figs. 8, 17). Like the connective-tissue sheet, the nerve layer is somewhat thicker in the bottoms of the gutters, beneath the lines of attachment of Class 1 fibers, but it never forms the thickest component of the stomach wall in *Patiria* as it does in some regions of the stomach in *Asterias forbesi* (Anderson, 1954: Fig. 10).

The epithelium lining the stomach presents no particularly remarkable features. Its typical tall columnar cells are covered distally by a brush border, and each cell bears a single long flagellum, arising from a conspicuous basal granule somewhat eccentrically placed in the distal end of the cell (Figs. 8, 18). Numerous mucous goblets are interspersed among the typical epithelial cells, with nuclei usually lying in the basal third of the cell and crowded to one side by the accumulated secretion (Figs. 6, 18). Other types of secretory cells are encountered very infrequently in the cardiac stomach proper; single cells, long and slender and containing a row or two of small secretory spherules, are occasionally found scattered at random among the cells of the general epithelium, but these are not at all common. In the region of the esophagus there is a considerable representation of secretory cells of a different type, tending to lie rather high in the epithelium and to have a somewhat bulbous appearance, packed and distended with coarse, deeply-staining spherules (Fig. 15). These I have interpreted not as endodermal zymogen cells, such as are abundant in the digestive diverticula (pyloric caeca), but as representing the so-called "mulberry cells" of the epidermis described by Cuénot (1887). Cells identical in appearance and staining behavior are numerous in the adjacent peristomial epithelium and gradually disappear in the esophagus; a very few may be encountered in the lowest part of the stomach.

A considerable degree of regional specialization, or consistent, patterned distribution of specific epithelial cell types, occurs in conjunction with the branching gutters of the stomach wall, although in *Patiria* this characteristic is never so marked as in *Asterias*. The flattened floors of the gutters are lined by comparatively low columnar cells with clear cytoplasm and small, rounded, granular nuclei (Figs. 17, 18). In the lower part of the stomach, where the gutters are narrow and deep, such cells appear only in their floors; higher in the stomach, where the gutters become broad and shallow, the columnar cells with small rounded nuclei occupy larger areas, increase in height, and constitute the most numerous class of epithelial cells (Fig. 21). The lateral walls of the deeper gutters consist of tall cells, very crowded, in which the nuclei are elongate and slender and stain densely. A narrow zone of transition marks the change from one type of epithelium to the other, just where the side walls rise from the floor (Figs. 3, 5, 16). The tall cells with dense nuclei are most numerous in the walls of the gutters; they continue over the lips of these grooves (Fig. 14) and gradually give way, in the areas between adjacent gutters, to equally tall cells with elongate but more granular and lightly-staining nuclei. Flagella are never multiple; each cell bears only one.

Mucous goblets are numerous in the floors of the gutters as well as among the taller cells in their side walls and near their lips. Small cells with spherical, granular nuclei, interpreted as amoebocytes, are commonly observed lodged between the epithelial cells, most conspicuously at levels above the epithelial-cell nuclei (Figs. 17, 18).

Although no detailed histochemical studies have been attempted, special technics demonstrate some additional characteristics in various histological components of the stomach. Glycogen appears to be very generally distributed throughout, with no notable points of concentration, as indicated by results of the periodic acid-Schiff routine. After removal, by salivary digestion, of all PAS reactivity attributable to glycogen, several sites retain strongly positive reactions. These sites include, as expected, the connective-tissue basement membrane of the stomach wall (Fig. 19) and related components of Class 1 retractor fibers, as well as the contents of mucous goblets and free mucus retained on the surface of the epithelium after release. In addition, the distal cytoplasm of virtually all the tall epithelial cells contains varying amounts of granular, finely-dispersed PAS-positive material (Fig. 19), the amount depending on the locations of the cells. Those in the side walls of narrow gutters usually contain the largest deposits; in other locations only a small amount of material just under the brush border, surrounding the basal granule of the flagellum, reacts positively. In addition to being PAS-positive, this material is basophilic (stains with Harris' hematoxylin—see Fig. 6) and exhibits a reddish metachromasia, persisting after alcoholic dehydration and mounting in resin, when stained overnight with dilute toluidine blue. These staining reactions are consistent characteristics of the distal deposits in tall cells with long nuclei; it is noteworthy that in the epithelial cells with rounded, granular nuclei, localized in the floors of deep gutters and more widely distributed in broad, shallow ones, the cytoplasm is always clear and never contains even a trace of such material. In contrast to the distal metachromatic material in the tall epithelial cells, the contents of mucous goblets, while similarly PAS-positive, lose their toluidine-blue metachromasia during alcoholic dehydration.

One further unusual feature of the cardiac stomach of *Patiria*, characteristic of all specimens examined, remains to be described, although its significance remains in doubt. In all levels of the stomach, the depths of the epithelial layer are occupied by numerous cystic growths, of various sizes. They may be small and nearly spherical, lying in the arcades between the basal processes of the epithelial cells just above the nerve layer (Figs. 16, 17, 18), or they may be much longer than their breadth and occur in such masses as to crowd the nuclei and major cytoplasmic portions of the epithelial cells into the upper half, or less, of the epithelial layer. The cysts are fully as variable in appearance as in size. They are always more or less completely filled with inclusions, which are either clear, non-staining, refractile spherules less than 2 μ in diameter; or slightly basophilic spherules of about the same size, each of which contains a single highly basophilic granule; or somewhat larger masses or groups of very small, deeply staining flocculent or granular bodies. The cysts appear to begin, at least, as intracellular bodies; in conjunction with the smaller ones a single large nucleus can usually be demonstrated, somewhat distorted and crowded to one side so that it resembles the nucleus of an active mucous goblet (Fig. 20). This nucleus is the only body in the cysts that gives a positive Feulgen reaction; although the small, highly basophilic granules within the inclusions otherwise stain like chromatin, they are Feulgen-negative. It seems reasonable to conclude, at least tentatively, that the cysts are of parasitic origin. A detailed study of this material would probably show that the inclusions in different cysts, so obviously different in appearance, represent successive stages

in the life-cycle of a sporozoan, widely distributed in *Patiria miniata* at Pacific Grove. Such a study is beyond the scope of the present investigation.

Discussion

Although the cardiac stomach of *Patiria* is very generally similar to that of *Asterias*, major differences in structural details appear when these organs are compared, as indicated briefly at several points in the preceding descriptive section. Close resemblance is not necessarily to be expected, as these genera are not closely related, belonging to different Orders; furthermore, their habits and feeding practices differ considerably. It is regrettable that comparably detailed studies have apparently not been made on *Asterina gibbosa*, a species very close to *Patiria* that has been more generally described by European investigators. It is of interest, however, to examine structural differences that exist between *Asterias* and *Patiria*, and to consider the functional differences to which they may give insight.

In view of the unusual degree and frequency of eversion of the cardiac stomach in *Patiria*, one might anticipate that provision of mechanisms for anchorage, reinforcement, and restraint of the everted vesicles, and for their rapid retraction, would be of the utmost significance. The existence of two supplementary systems of fibers here, in addition to the branching intrinsic retractor system (Class 1 fibers) found also in *Asterias*, is undoubtedly related to the functional demands of this special situation. Unfortunately, experimental evidence as to the contractility and behavior of these fibers in *Patiria* is not available; in its absence, deductions as to their probable functions may be made from the nature, histological composition, and anatomical relationships of the several fiber systems.

Class 1 strands are stout mixtures of connective-tissue and muscle bundles, anchored proximally to the extrinsic retractors (of which they are actually continuations), attaching all along their lengths by broad insertions on both the connective-tissue and muscle layers of the stomach wall, and extending to widespread terminal attachments at the extreme lower end of the cardiac stomach. These are almost certainly the principal restraining and retractor bands of the stomach; contractions in these strands would be very widely transmitted to the everted vesicles and would be most effective in compressing them to force the coelomic fluid back into the perivisceral cavity, and in drawing the collapsed folds uniformly back through the mouth. It is true that the contractility postulated by Anderson (1954) for the supposedly homologous intrinsic retractors in *Asterias* could not, after all, be experimentally demonstrated (Burnett and Anderson, 1955). In *Patiria*, however, the Class 1 strands contain a much greater complement of muscle fibers than the corresponding structures in *Asterias* and so may be considered more likely to be contractile.

Muscular contractions in Class 1 fibers may be transmitted even more broadly, in areas to which their branches do not extend, through the mediation of the Class 2 fibers. These originate in the muscle layer on ridges of the stomach wall, and the muscle fibers of which they are entirely composed may actually be traceable to the contractile components of the adjacent Class 1 strands. At their other ends, they distribute their fibers very widely in the muscle network of the stomach wall. In addition to maintaining the gutter-patterns in the lower part of the stomach, the

Class 2 fibers are of such composition and anatomical relationships that they could serve very importantly in collapsing the everted vesicles of the stomach at retraction, or simply in aiding the muscle layers of the stomach wall to resist excessive stretching in response to increased internal pressure.

In contrast, the Class 3 fibers, consisting solely of single strands of fibrous connective tissue, must function in a purely mechanical fashion. The myriads of short, parallel fibers that both begin and end in the connective-tissue layer of the stomach wall can only reinforce and restrain the extreme lower end of the stomach where it joins the peristome. The coarser, longer strands, originating in smaller numbers in the connective-tissue bundles of Class 1 fibers, must function to transmit tension from these to the lower stomach wall, in areas not reached by either Class 1 or Class 2 branches.

Little can be said concerning the functions of the extrinsic retractor harness. It is of interest, however, to note that the fan-shaped bands, of which there are two in each ray, are not of such simple construction as those in *Asterias* but are composed of groups of fibers with different relationships. The strands that continue on the stomach wall as branching Class 1 fibers are probably contractile, as their continuations almost certainly are. It is difficult to see how contractility in the strands connecting the circumferential girdle of the stomach to the ambulacral ossicles could serve any useful purpose; shortening of these strands would dilate the passageway between cardiac and pyloric stomachs and perhaps depress the floor of the pyloric stomach, but the contribution of such an action to the mechanism of eversion or retraction of the stomach is difficult to evaluate. At any rate, until experimental evidence as to the possible differential behavior of these various strands is available, further speculation is fruitless.

It has been remarked that regional specialization of the tissue components of the stomach wall is much less pronounced in *Patiria* than in *Asterias*, although the gutter-patterns with which this is associated are about equally well developed in the two forms. It is particularly noteworthy that the ridges between gutters in *Patiria* are not clothed with tall cells containing huge, densely-staining, cigar-shaped nuclei and bearing multiple flagella so characteristic of such regions in *Asterias*. A relatively small number of cells localized here in *Patiria* do contain long, comparatively dense nuclei, but the difference between these and their counterparts in *Asterias* is striking. If such cells represent sensory receptors, as Smith (1937) suggested for *Marthasterias glacialis*, then *Asterias* is obviously better supplied with gastric sense organs than is *Patiria*. Yet the stomachs of the two forms are apparently equally delicate and susceptible to injury and ought to be equally sensitive to stimuli from hazardous situations during eversion. The observed differences may simply be related to differences in the general characteristics of forms in the two Orders to which these species belong; or it may be that different, as-yet-unrecognized cells in the stomach of *Patiria* are functioning as sensory receptors. On the other hand, it may be suggested that the observed differences reflect the marked contrast in feeding habits exhibited by *Asterias* and *Patiria*. Although the stomach of *Asterias* is less frequently and less broadly everted than that of *Patiria* and hence perhaps not so often exposed to the general vicissitudes of the external environment, it is very often insinuated through the minute gape between the shells of living bivalves (Burnett, 1955; Lavoie and Holz, 1955; Lavoie, 1956)

and must be provided with patches of specialized sensory epithelium for its guidance and protection. Feder (1955) has reported similar behavior in the stomach of *Pisaster ochraceus* attacking clams and oysters; and in his experiments with *Evasterias troschelii*, Christensen (1957) found that although the tube-feet of this species might often be trapped and cut off between the closing valves of an imitation clam, folds of the cardiac stomach never were. Patches of specialized epithelium closely resembling those found in the stomach of *Asterias* are very well developed also in the cardiac stomachs of *Pisaster ochraceus* and *Pycnopodia helianthoides* (personal observations, unpublished); I have no information on *Evasterias*, but the occurrence of such "sensory" patches in the stomachs of *Asterias*, *Marthasterias*, *Pisaster*, and *Pycnopodia* suggests that this may be a common characteristic of members of the Family Asteriidae, related to their habit of inserting the everted cardiac stomach into living bivalves. According to my observations, the stomach of *Patiria* operates in no such sophisticated manner; it is merely everted as broadly as possible and wrapped about whatever objects of food are available, or perhaps used as a flagellary-mucous feeding organ.

Experiments using sea-water suspensions of Congo-red-stained yeast cells or India ink to trace currents over the surface of the stomach reveal that the gutters do not, as might have been expected, serve to conduct food-bearing currents upward into the stomach. Such currents as do appear in the vicinity of the grooves are directed in such a way as to move material from the depths of the grooves upward over their lateral lips and thence away from the surface of the epithelium; there appear to be no longitudinal currents, either upward or downward, in the gutters themselves. The action of flagella on the ridges between gutters does produce currents carrying particles upward into the stomach. These observations are essentially in agreement with those reported for *Asterias forbesi* (Anderson, 1954).

Digestion of food materials of all kinds surrounded by the everted cardiac stomach or drawn into its cavity after retraction is rapid and complete. A small specimen of *Patiria* (radius about 2 cm.), trapped against the glass of an aquarium by a large individual (radius about 5 cm.) and enveloped in the folds of its everted stomach, was reduced within a day and a half to a small heap of dissociated skeletal ossicles; examples of this kind could be multiplied indefinitely, all testifying to the powerful nature of the digestive juices acting in the cardiac stomach. In view of this, the extreme paucity of anything resembling zymogen cells in the epithelium of the cardiac stomach is surprising and raises the suspicion that perhaps digestive enzymes are being produced by cells in the stomach lining that do not resemble those customarily identified as the sites of enzyme production in starfishes (Anderson, 1953). An alternative explanation, more in line with classical interpretations of asteroid digestive processes, would hold that the stomach itself produces no enzymes but serves only as the organ which envelopes the food and mixes it with enzymes brought to it from the myriads of typical zymogen cells in the pyloric caeca.

The true state of affairs with regard to these questions was determined by simple experiment. All 5 pairs of pyloric caeca were operatively removed from a large specimen without interfering with cardiac stomach, pyloric stomach, or other parts of the digestive tract; the specimen was then allowed to recover for a period of 8 weeks. Well before the end of this time the integrity of the body wall had been restored by healing of the incisions, and with it the ability to evert the cardiac

stomach returned. After 8 weeks, the operated specimen was offered a large, distinctively-colored piece of liver and ovary from a snail, which it readily ingested. As a control, a similar piece of snail tissue was fed to a normal starfish of the same size. After 24 hours, the cardiac stomach of the control animal contained only a semifluid, disorganized *brei,* while the piece of snail tissue in the stomach of the operated starfish remained intact and in fact retained its original color. Dissection of this specimen revealed that regeneration of the pyloric caeca had begun but had advanced only to the extent of producing short, simple, tubular rudiments with none of the highly specialized features characteristic of the normal organs, and so presumably without any considerable population of zymogen cells. This experiment demonstrates that the stomach of *Patiria,* which displays an almost complete lack of granular secretory cells, does not secrete digestive enzymes, or at least does not produce them in quantities sufficient to bring about normal digestion. The pyloric caeca, which contain very large numbers of granule-filled cells interpreted as zymogen cells, are obviously necessary for the production of digestive enzymes; in the absence of these organs, digestion does not occur. The enzymatic secretions which they normally produce are carried to the cardiac stomach by flagellary currents, described for this species by Irving (1924), forming a part of the regular circulation that also brings the products of digestion from the stomach into the pyloric caeca.

Many other aspects of structure and function in the digestive system of starfishes await elucidation; detailed anatomical and histological studies, such as those reported here for a limited portion of the alimentary system in a single species, still remain to be done for a majority of even the commonest asteroids. Such studies, continued and broadened, will make possible meaningful comparisons between closely and distantly related species, and between species of diverse habits and ways of life. Most importantly, detailed and accurate anatomical studies serve as a basis for correlation and interpretation of the results of experimental physiological studies which can, in turn, furnish explanations for anatomical details.

Summary and Conclusions

The large and voluminous cardiac stomach of *Patiria miniata,* frequently and very extensively everted in the normal feeding behavior of this omnivorous intertidal starfish, is provided with an unusually elaborate retractor harness. As in *Asterias,* this consists of an extrinsic portion extending from the ambulacral ossicles to the stomach, and an intrinsic portion spreading over the stomach in a regular pattern of branching strands, here designated Class 1 fibers. A second system consists of small, muscular strands extending horizontally in the region of the lower Class 1 branches and inserting on the muscle layer of the stomach; these have been termed Class 2 fibers. Still a third set, composed of single strands of connective tissue, arise either from Class 1 fibers or from the connective-tissue layers of the wall of the stomach, take a short, straight, longitudinal course downward, and insert again on the connective-tissue sheet in the stomach wall. These Class 3 strands occur in large numbers, limited to the extreme lower end of the stomach. It is assumed that all these accessory components of the retractor harness have arisen in connection with special problems presented by the size and mode of operation of the cardiac stomach; the several systems of strands, varying in com-

position and in anatomical relationships, undoubtedly perform specific, different functions in reinforcing, restraining, and retracting the spreading vesicles of the cardiac stomach.

The wall of the stomach has essentially the same histological composition as that usually found in the asteroid digestive tract, with connective-tissue and nerve layers showing localized thickenings in the vicinity of Class 1 fibers. These strands are also accompanied by correspondingly branched, specifically distributed patterns of folds and ridges forming gutters in the stomach wall. Consistent patterns of cell localization in the epithelium lining the stomach are related to the gutters; comparatively low cells with rounded nuclei line their floors, while their side walls are clothed by taller cells with long, dense nuclei. Mucous goblets are common, but there are practically no granular secretory cells. The regional specializations in the distribution of epithelial-cell types in *Patiria* are much less marked than in *Asterias;* in particular, *Patiria* lacks the conspicuous patches of tall cells with huge, cigar-shaped nuclei and multiple flagella (thought to be sensory receptors) localized between the gutters in *Asterias*. This may indicate that the cardiac stomach of *Patiria* is less sensitive to external stimuli than that of *Asterias;* possible explanations for this may involve differences in feeding habits and the behavior of the stomach between these two types of starfishes.

The cardiac stomach of *Patiria* lacks zymogen cells almost completely; this is surprising in view of the rapidity and versatility of the digestive activities carried on by this organ. It has been experimentally demonstrated, however, that individuals operatively deprived of their pyloric caeca are unable to digest food; although they ingest it normally and hold it in the cardiac stomach, food remains intact for at least 24 hours, a period sufficient for almost complete breakdown of similar food in the stomach of a normal animal. It is concluded that the myriads of granular secretory cells in the pyloric caeca, usually interpreted as zymogen cells, are the source of the digestive enzymes acting to bring about normal digestion of food in the cardiac stomach.

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THE BREEDING SEASON OF THE BRACHIOPOD, LINGULA UNGUIS (L.)

S. H. CHUANG

University of Malaya in Singapore, Singapore

Yatsu (1902) believed that the breeding season of *Lingula unguis* lasted from mid-July to the end of August in Misaki, Japan, since he could not find the larvae at any other time of the year. Kume (1956) confirmed Yatsu's observation, and also found that the peak of breeding occurred during the first half of August. Sewell (1912) found several larvae in December and February in the plankton off the south coast of Burma. He attributed this (p. 90) to "either a local peculiarity or possibly to the existence of two breeding seasons during the year, one in the summer months July and August, and a second from December to February." Ashworth (1915) obtained larvae in the southern part of the Red Sea in June and October, and in the Indian Ocean in October. He also found a larva in Annandale's plankton sample obtained in the Strait of Bab-el-Mandeb in March. This led him to believe that in the southern part of the Red Sea a succession of spawnings extended at least over the period from the beginning of March to the early part of September.

Yatsu (1902) observed the spawning of *Lingula unguis* in captivity on several occasions in July and August. He noted that spermatozoa and ova were discharged forcibly through the median setal tube. Kume (1956) observed that in *L. unguis* spawning in the laboratory occurred twice daily, at sunrise and after sunset.

In the present study plankton samples were collected to determine the occurrence of the larvae of *Lingula*. The spawning behavior of adult female specimens in the laboratory was also studied.

Materials and Methods

The plankton hauls were made with a fine tow-net at a depth one foot below the surface off the north coast of Singapore Island. On each occasion 4–10 hauls of 10 minutes each were made, more hauls being made under favorable weather conditions. The *Lingula* larvae from the plankton samples were isolated and classified according to the number of pairs of cirri. In this study a larva with "n" pairs of cirri and a pair of buds, the length of each of which has equalled the diameter of the base of the median tentacle, is assigned to the stage of "n + 1" pairs of cirri (abbreviated to n + 1 p.c. stage). Although the hauls were made 1–2 miles from a good bed of *Lingula unguis*, it was not possible to assign the larvae to this species with certainty.

Specimens of *Lingula unguis* for the laboratory study of spawning behavior were obtained from the north coast of Singapore Island. A plot of muddy sand with the greatest concentration of burrows was chosen, and from it all the available

specimens were dug out with a shovel during ebb-tide. The area worked roughly amounted to 5–15 square meters per trip. In the laboratory every 25 specimens were laid on their dorsal or ventral side in a rectangular tray, 30 cm. by 45 cm., under 4 cm. of natural sea water renewed once a day. These trays were stacked on shelves in a darkened room maintained at a temperature of 18–20° C. Every morning they were examined with the light of a lamp for spawned ova. Specimens that had spawned were transferred into marked petri dishes, 9–20 cm. in diameter and with a capacity of 50–400 cc. They were maintained in these dishes with daily renewal of water during the rest of the experimental period for easier estimation of the ova spawned.

Frequent attempts were also made to find the time of settlement of the larvae by searching for the young postlarvae at the *Lingula* beds along the north and east coasts of Singapore Island.

Results

I. Occurrence of planktonic larvae

The larvae of *Lingula* obtained from plankton hauls during the period July, 1952–June, 1953 are tabulated according to the number of paired cirri. Dead larvae with disintegrating cirri are assigned to the column of unclassifiable specimens (Table I).

Inspection of Table I shows that (1) the larvae appeared in practically every month of the year, suggesting a continuous breeding throughout the year. This fits in with Orton's rule (1920, p. 353), "that in those parts of the sea where temperature conditions are constant or nearly constant, and where biological conditions do not vary much, that marine animals will breed continuously." (2) On most occasions the free-swimming larvae were at different stages of development. This suggests that the larvae of each catch were not the products of any single day's spawning. (3) Older larvae of 8 and 9 p.c. stages were rare. (4) Young larvae of 2 and 3 p.c. stages appeared in July, August, September, October and November of 1952 and in January, February and June of 1953. (5) The young larvae of 2 and 3 p.c. stages were captured two or three times at intervals of 5–7 days in August and September. Their occurrences did not bear any relationship to the phases of the moon.

II. Laboratory spawning of the females in Lingula unguis

Many specimens brought into the laboratory in every month of the year were found on dissection to have ripe ova. Observations of laboratory spawning are summarized in Table II, which shows that (1) spawning occurred 5–14 days after collection among specimens collected on every occasion. (2) Observation on the duration of spawning of each batch was completed only for the batch collected on July 29, 1952. Observations on other batches were discontinued after various periods. (3) The smallest specimen that spawned had a ventral-valve length of 22.6 mm. and the largest, 46.0 mm. Presumably, the females become sexually mature in this locality when they attain the former size approximately, and continue to spawn thereafter at intervals. The presence of ripe ova in many specimens exceeding 50 mm. in length seems to indicate that the spawning power is retained in old age.

TABLE I

Number of Lingula larvae from plankton samples obtained off the north coast of Singapore Island

Date of collection	Number of larvae with the following pairs of cirri									Total
	2	3	4	5	6	7	8	9	Unclassifiable	
14. 7.1952		28	2						1	31
22. 7.1952					1					1
13. 8.1952	10	5	3						2	20
19. 8.1952	20	31								51
24. 8.1952		4	81	9	1	3			1	99
31. 8.1952										0
7. 9.1952										0
14. 9.1952			2							2
21. 9.1952	4	59	21	1						85
28. 9.1952	3	14							12	29
6.10.1952										0
26.10.1952	9	38		1	6	1				55
2.11.1952										0
9.11.1952										0
16.11.1952		10	39							49
6.12.1952										0
15.12.1952				1	1				3	5
3. 1.1953	80	15	2		4	6	3			110
2. 2.1953		4							6	10
26. 3.1953									2	2
15. 4.1953										0
20. 5.1953			6	1						7
27. 5.1953				1	4	2		1		8
12. 6.1953		2		6	59	115	12		25	219
29. 6.1953						4	8	1		13
Total	126	210	156	20	76	131	23	2	52	796

TABLE II

Laboratory spawning of Lingula unguis from the north coast of Singapore Island

Date of collection	Onset of spawning (days after collection)	Total spawning days, when expt. is discontinued	Number of males and females collected	Number of spawning females	Ventral-valve length of spawning females (mm.)	
					Minimum	Maximum
30. 5.1952				A few		
28. 6.1952	6	74	159	55	23.9	41.0
14. 7.1952	8	95	134	6	30.3	39.1
29. 7.1952	5	188	293	57	22.6	46.0
28. 8.1952	5	78	275	28	23.4	43.2
13. 9.1952	14	52	93	11	27.5	37.1
6.10.1952	12	82		11	27.0	42.3
5.11.1952	6	80	59	9	27.5	38.3
30.11.1952	11	59	65	9	26.7	39.0
31.12.1952	10	29	316	10	32.4	43.4
27. 2.1953				A few		

Further data on the batch collected on July 29, 1952 are summarized in Figure 1, which shows that (1) during a period of 6 months or so, there were some specimens spawning each week. (2) The most intensive spawning occurred between the seventh and thirteenth week of captivity when from 109–135 cases of spawning per week were observed. (3) Cases of intensive spawning when several thousand ova were extruded by a female per day occurred during the first half of the observational period.

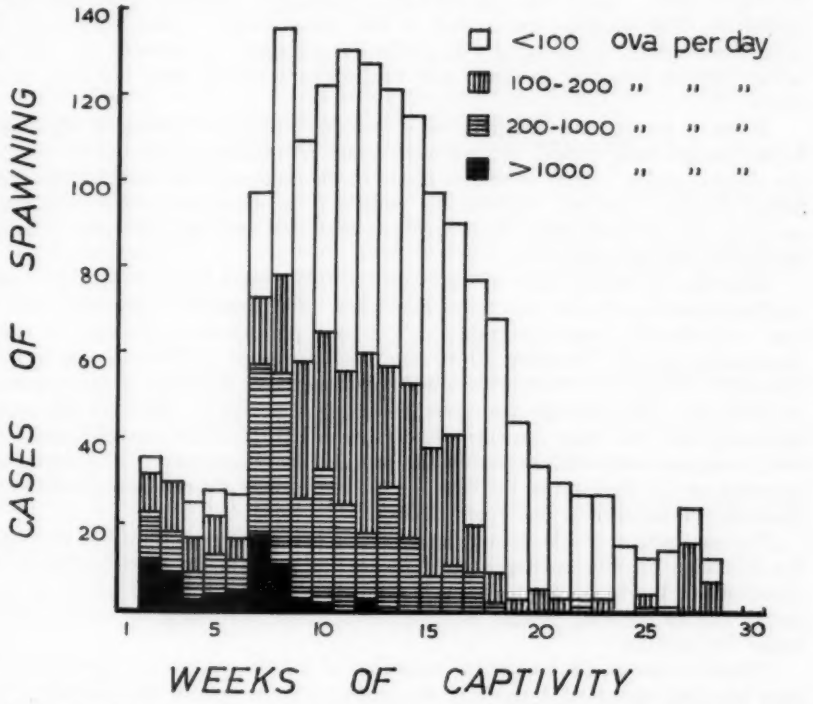

FIGURE 1. *Lingula unguis*. Number of cases of laboratory spawning per week during captivity. These cases were compiled from daily records of fifty-seven spawning females collected on July 29, 1952 from the north coast of Singapore Island.

The spawning behavior of 5 females from among the more consistent spawners of the July 29, 1952 batch reveals that (1) all the ova of an individual were not extruded at once. (2) Spawning in a female occurred in a series of bursts separated by rest intervals. (3) The first burst was usually the most intensive and consisted of 1–23 days of spawning. (4) Other bursts of lower intensity followed at irregular intervals over a period of 2–3 months. (5) The laboratory spawning did not have any relationship to the phases of the moon or the tides.

Spawning occurred both day and night. The ova were forcibly squirted out of the central exhalant setal tube, as Yatsu (1902) had previously observed. On

a few occasions these came out in the accessory exhalant currents found midway along the lateral gape of the animal. In 24 hours a spawning female produced from half a dozen to about 3000 ova. When only a few ova were spawned, these were found singly in the dish. However, when a few hundred or more ova were extruded, they were coated liberally with mucus which enabled them to stick together and form big sheets or clumps, as Yatsu (1902) had noted.

The newly spawned ovum was spherical in shape. The mean of 62 ova was $95.55\,\mu \pm 4.60\,\mu$. Each was enclosed in a prominent vitelline membrane $3\,\mu$ in thickness. The nucleus was visible in the living ovum. After some time the unfertilized ovum degenerated. It gradually enlarged, presumably on imbibing water, became irregular in shape and finally disintegrated after 1–2 days in sea water.

When a transparent specimen was examined under a binocular microscope during its spawning period, the ova were found circulating in the coelomic fluid in the visceral cavity. They were also found in the coelomic fluid in the longitudinal pallial sinuses. On one occasion the pedicle of a spawning female accidentally snapped; the coelomic fluid that oozed out contained not only the usual coelomic corpuscles but also some ova.

Analysis of further data of the July 29, 1952 batch reveals that (1) large specimens produced more ova than small ones. For instance, 6 females of 41–46 mm. ventral-valve length averaged 17,250 ova per specimen during the entire observation period; 9 females, 30–40 mm. long, averaged 13,000 ova with a maximum of 17,800 ova; 3 others, all below 30 mm., averaged 4000 ova with a maximum of 7500 ova. (2) During the entire observation period of 188 days the largest specimen, 46.0 mm. long, spawned 28,600 ova in 104 days; the second largest, 43.5 mm. long, spawned 22,350 ova in 76 days. (3) A specimen, 39.8 mm. long, spawned on 125 days out of 188 days. (4) The females dissected at the end of the spawning period usually had spent ovaries.

The production of a large number of ova is presumably necessary to counteract the following possible wastages: (1) wastage of ova through fertilization taking place outside the body, when the germ cells are shed into the sea; (2) wastage of larvae due to the long pelagic larval period, which, according to Yatsu (1902). lasted 1½ months.

Observations on the laboratory spawning of female specimens suggest that the long breeding season of *Lingula* in the tropics may be due to the following: (1) all the ova were not shed at once but intermittently over a long period of time; (2) the staggering of the peaks of individual spawning in a large population; (3) the presumable tendency of the postlarvae from the different spawnings to reach spawning age at different times of the year.

The female specimens in *L. unguis* continued to spawn in the laboratory in complete isolation from the males, contrary to the hypothesis of Yatsu (1902, p. 4) that "Until the sperm is discharged the eggs even when well ripened seem to be retained within the body."

The females, even when reared singly in separate petri dishes, continued to spawn in the laboratory for several months. Crowding therefore was not necessary for the females to continue spawning, contrary to the observation of Kume (1956, p. 223) that "the frequency of shedding was greatly reduced in the vessel in which only a small number of individuals were cultured."

III. Occurrence of young postlarvae

Attempts to collect the young postlarvae were unsuccessful in the muddy *Lingula* beds along the north coast of Singapore Island. Along the east coast the beds are sandy. Here some young postlarvae with the following minimum ventral-valve lengths were obtained on the following dates: April 16, 1952, 6.5 mm.; September 24, 1952, 2.7 mm.; October 22, 1952, 3.7 mm.; November 3, 1952, 5.8 mm.; and July 3, 1953, 1.4 mm. Presumably it was only when the spatfall was considerable that the young postlarvae could be found.

Summary and Conclusions

1. The planktonic larvae of *Lingula* were found in almost every month of the year, suggesting continuous breeding off Singapore Island.
2. Several heavy spatfalls were observed in 1952, indicating several peaks of spawning during the year.
3. The spawning behavior of the female in *Lingula unguis* was described and its bearing on the continuous breeding in the tropics was suggested.
4. It was suggested that a female spawned a large quantity of ova to counteract the possible wastage of ova accompanying external fertilization and the wastage of larvae during the long pelagic larval period.

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NUCLEAR SIZES IN RANA MESONEPHROI [1]

MARY GRACE CONNELL [2]

Biology Department, The Catholic University of America, Washington, D. C.

When Jacobj (1925) devised the now classical caryometric method there was opened up for the cytologist a whole new field in population dynamics. Jacobj demonstrated that for any given organ the nuclear population showed considerable variation in size from one individual nucleus to another. By plotting frequency distribution curves of nuclear volumes he obtained evidence to show that the volumes increased discontinuously according to a logical pattern. The peaks of the curve corresponded to nuclear volumes which when arranged in series gave the geometric progression 1:2:4:8. Nuclear class series have since been described for a wide range of both invertebrate and vertebrate tissues.

Caryometry has been used extensively in investigations of ploidy, of endomitotic growth, and of the interphasic growth of nuclei in a dividing tissue. The concept that nuclear size is a function of ploidy has proved fruitful in the study of ploidy in amphibians (*cf.* Fankhauser, 1945; Gallien, 1953). This idea was used advantageously in the interpretation of polymodal curves obtained from nuclear volume data derived from studies of the kidneys of frogs: it was indicated that polysomaty may occur in this organ (Dawson, 1948; Schreiber and Melucci, 1949). Polysomaty or endopolyploidy is understood in this paper as that condition existing in a normal diploid somatic tissue in which there is a certain percentage of polyploid cells and/or polytene chromosomes. Furthermore, the concept that nuclear size is a function of chromosomal reduplication has been helpful in the interpretation of data having to do with interphasic growth of nuclei in a dividing tissue. Nuclear class series indicative of a mitotic cycle have been described in *Ambystoma* larvae (Swift, 1950) and in *Rana pipiens* embryos (Sze, 1953). Both of these investigations were primarily concerned, not with relationships in size, but with the photometric determination of amounts of desoxyribose nucleic acid (DNA) in interphasic nuclei. The introduction into cytology of photometric techniques has renewed interest in the caryometric interpretations of nuclear sizes.

Nuclear size as a reflection of ploidy relates importantly to amphibian development both in regard to gross morphology and in regard to tissue differentiation (Fankhauser, 1945; Gallien, 1953). Also it has been shown in certain molds that both cell size and nuclear size changes may accompany morphogenesis (Bonner, 1957). Furthermore, it has been hypothesized that DNA may show a slight decrease with the progressive differentiation of certain *R. pipiens* tadpole tissues (Moore, 1952). Also of importance is the fact that nuclear size may be related to the degree of functional activity of the cells in question. For instance, spinal

[1] A paper submitted to the faculty of the Graduate School of Arts and Sciences of the Catholic University of America in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Present address: College of New Rochelle, New Rochelle, New York.

cord cells of *R. temporaria* show a decrease in volume following narcosis (Krantz, 1947). On the other hand, nuclear size may increase as a result, not of chromosomal increase, but as a result of protein synthesis in the nucleus. This has been shown to be true for certain insect and mammalian tissues by Schrader and Leuchtenberger (1950) and by Leuchtenberger and Schrader (1951).

Nuclear size is, then, of considerable importance in the investigations of some of the most fundamental biological problems. The concepts and principles discussed here have not been extensively applied to nuclear size relationships of specific organs during the metamorphic stages of amphibians. Investigators have concentrated mainly on embryos before metamorphosis and on adult tissues. This paper is concerned with these concepts and principles by way of the study of the mesonephroi of *Rana sylvatica* during metamorphosis by the use of the classical methodology of caryometry.

Materials and Methods

Two clutches of *Rana sylvatica* eggs were collected in the field and were allowed to develop in the laboratory throughout an eighty-day period. The metamorphic stages selected for study at arbitrary time intervals were identified by means of the criteria established by Taylor and Kollros (1946) for *R. pipiens*. Some of the tadpoles were carried through metamorphosis to young adulthood. Table I gives both stage and age for all animals used in this investigation. Immediately after the staging of the tadpole the body cavity was opened and the animal was immersed in Zenker-formol for fixation. After fixation the mesonephroi were removed, embedded in paraffin and sectioned either transversely or longitudinally at 6 micra. All sections were stained by the Feulgen technique after Stowell (1945).

Only the center sections of a kidney from each of the 63 animals were examined. From these sections an average of 470 camera lucida outline drawings of nuclei from the convoluted tubules were made at a magnification of 1350 × diameters. The selection of nuclei to be drawn was random. However, it was necessary to set up certain criteria as a basis for selection. The first criterion was established in the following manner. In order not to cause marked distortion in the data the outline drawing of a nucleus was made at the focus showing the greatest nuclear diameter. If the nucleus is sectioned it is impossible to determine whether or not the greatest diameter lies in the section of tissue studied or in the next succeeding section. Therefore, nuclei lying in the two cut surfaces had to be eliminated on this basis. If it was possible to focus on the tissue at a level above and at a level below the nucleus selected, then it was certain that the entire nucleus was in the section. In establishing the second criterion, the long and short diameters were determined in mm. for each drawing. From these data the nuclear volumes were calculated according to the formula for the volume of an ellipsoid. It is a fact that this volume is not often used in similar studies of biologic material because of the difficulty of determining a diameter in the third dimension. This is practically impossible in sectioned material. However, in the kidneys of *R. sylvatica* used in this study the nuclei appear more or less spherical both in cross-section and in longitudinal section. On the basis of observation, therefore, it was concluded that most of the nuclei were not markedly flattened. Any nucleus that was definitely elliptical from surface view was eliminated. Also, any nucleus that appeared obviously thin on focusing through it was also eliminated. This device is, of course, arbitrary and subjective and does not remove the difficulty of the third diameter.

TABLE I

The table shows the modal volumes in mm.³ for the peak classes, and the ratios × x/766 mm.² where x is any one of the modal volumes and where 766 mm.³ is the lowest volume in the table

Stage	Class I nuclei	Intermediate class	Class II nuclei	Age in days
3	949(1.2)	1288(1.7)		18
4		1310(1.7)	1762.5(2.3)	24
		1290(1.7)		
5		1069(1.4)		22
		1226.5(1.6)		
		1075(1.4)		
6	766(1.0)	1105(1.4)	1378(1.8)	32
7		1225(1.6)	1364.5(1.8)	39
		1114(1.4)		
8		1231(1.6)	1400(1.8)	32
			1363(1.8)	
9		1066(1.5)	1369(1.8)	39
			1358.5(1.8)	
10		1270(1.7)	1500(1.9)	37
		1229(1.6)		
11–12		1130(1.5)	1511.5(2.0)	36
		1028.75(1.3)		
13–15	952(1.2)	1143(1.5)		36
		1061(1.4)		
16–17		1248(1.6)	1513(2.0)	39
		1138(1.5)		
18		1152(1.5)		43
		1294(1.7)		
		1109.5(1.4)		
19			1375(1.8)	45
			1369(1.8)	
			1355(1.8)	
20		1246(1.6)	1376.5(1.8)	47
			1355.5(1.8)	
21		1114(1.4)	1443(1.8)	47
			1381.25(1.8)	
22			1512(2.0)	50
			1926(2.5)	
			1811.5(2.3)	
23		1054(1.4)		57
		1334(1.7)		
		1300(1.7)		
24		1320(1.7)	1384(1.8)	57
		1160(1.5)		
*25	775.5(1.0)	1087(1.4)	1519(2.0)	80
	846(1.1)	1097.5(1.4)	1522(2.0)	
	958(1.2)		1417.75(1.8)	
			1349(1.8)	
			1433.5(1.9)	

* Young adults.

In addition, there are difficulties other than those due to diametric differences. For instance, there are a number of sources of error, not the least of which are those inherent in the investigator. The eye in seeing and the hand in drawing are not always coordinated to the same degree. There is the problem of focusing on

the maximum nuclear surface, the possibility of inaccuracy in measurement, the effect of the procedures on the nuclei themselves, and according to Merriam and Ris (1954) the probable sources of error in the camera lucida projection method. Further, there is the fact that the error due to method has been raised to a higher power in the formula for determining volumes. However, a search for absolute values and statistical certainty is not the purpose of this paper. Only a relative

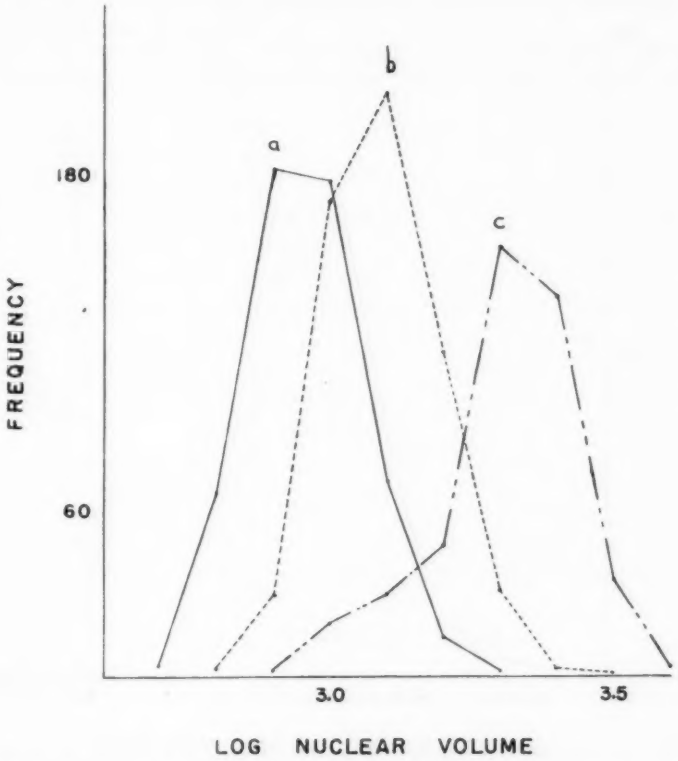

FIGURE 1. Histograms of three samples, one from each of three stages: a) stages 6, b) stage 18, c) stage 22.

value or an approximate value is sought. No attempt has been made here at statistical analysis of the data.

After determination of the nuclear volumes, these values were grouped into frequency classes and frequency curves were drawn for each one of the 63 kidneys. The modal volume for the peak class was determined for each case and they are recorded in Table I. This table shows that the lowest modal volume (766 $mm.^3$) occurs in one of the individuals at stage 6. Using this volume for comparative

purposes a ratio can be obtained for each modal volume listed. The ratios form a progression, 1 : 1.5 : 2, with nearly all intermediates between these values.

In the histograms the frequency of nuclear volumes is plotted against the class interval. Also, one third of the data were plotted by two other methods. In the one case, the long diameters of the nuclei were substituted for nuclear volumes; in the other, the logs of the nuclear volumes were plotted against frequency. The long diameters or the surface areas are frequently used as a substitute for volume

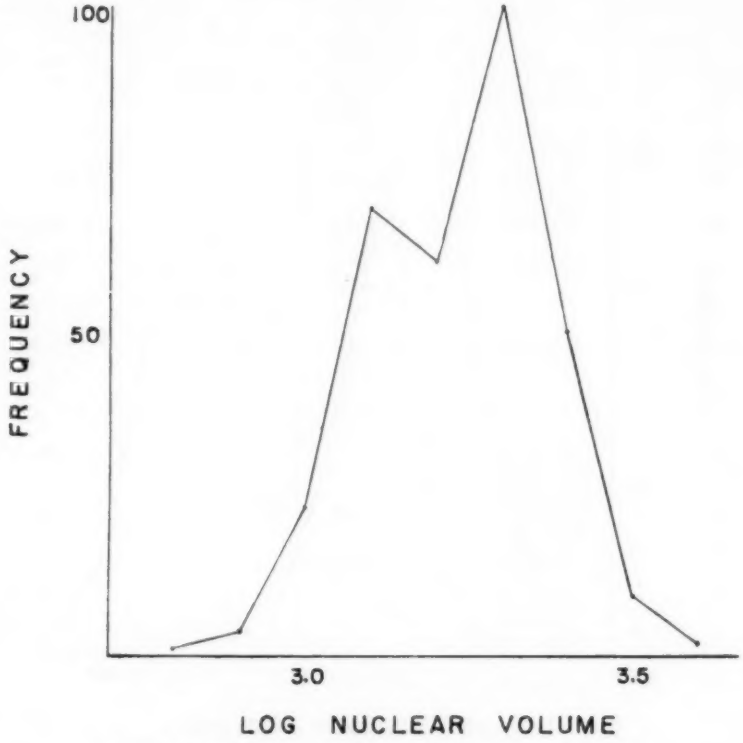

FIGURE 2. Histogram of one of the individuals at stage 24.

when the nuclei are ellipsoidal. Both values have the advantage over volume calculations since it is not necessary to multiply the error by raising the diameters to a higher power. In this study one third of the samples were selected and frequency curves were drawn using long diameters rather than volumes. In all cases the curves correspond to those obtained in histograms in which numbers were plotted against volumes. It appears, therefore, that for the data presented here there was no great advantage of one method over the other, at least in terms of the revelation of three classes of nuclei. If, however, a curve of greater symmetry is

desired then the use of logarithms of nuclear volumes has a distinct advantage over both volume and the long diameter. The logs of nuclear volumes give a more symmetrical curve than the nuclear volume itself (Figs. 1 and 2). Bucher (1954) claimed that the logarithmic system of classification is better adapted to a mathematical analysis of the curve and that it is the only valid system from a biological and statistical viewpoint. On the other hand, it has been stated (Bonner and Eden, 1956) that a mathematical analysis of the curve is not especially helpful for comparative purposes except in those restricted cases where there is additional information about cell or nuclear growth.

Finally, the geometric mean of the nuclear volumes was calculated for each kidney for purposes of plotting the logarithmic growth curve (Fig. 3). The geo-

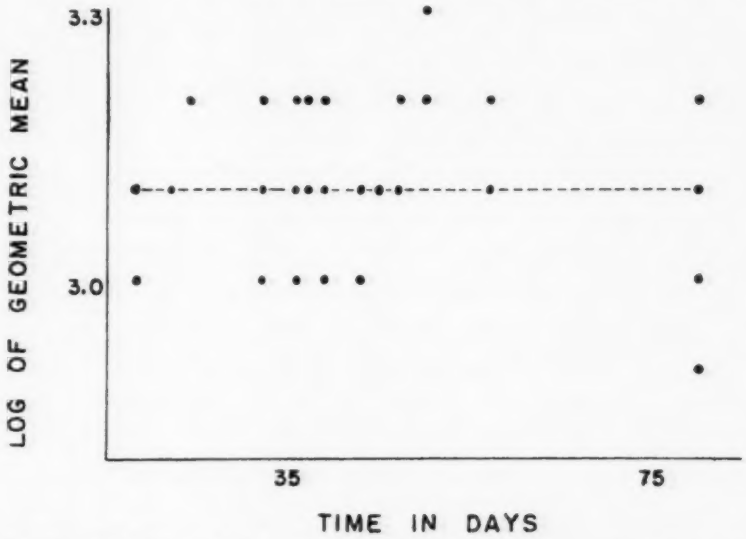

FIGURE 3. Logarithmic growth curve. Each point plotted represents from one to four individuals. Thus, at 80 days the 10 young adults are represented in the four points.

metric mean is subject to all those errors inherent in the method and is, like the nuclear volume, an approximation.

OBSERVATIONS

The frequency distribution curves of all but one of the kidneys studied show a single peak and are therefore unimodal. There is one bimodal curve (Fig. 2) in which the nuclear volumes give two peak classes. In all cases, when the modal volumes (Table I) calculated for these peaks are arranged in a series they form a progression in the ratio 1:1.5:2. Therefore, the histograms reveal two major classes and one intermediate class (Fig. 1).

In Class I the nuclei are believed to have the diploid value characteristic of post-

mitotic nuclei. Following division there is, possibly, a period of interphasic growth of the chromosomes during which the nuclear volumes increase to the size of the intermediate class. This may be followed by a second period of interphasic growth during which the nuclear volume becomes twice that of the diploid nucleus. Nuclei of Class II may, therefore, be tetraploid and ready for division. All intermediate values shown in Table I are interpreted hypothetically as intermediate classes which would be expected to appear during the two interphasic growth periods. The three peak classes are shown in Figure 1 where three of the 63 histograms were selected for illustrative purposes. It is to be noted that in both Figures 1 and 2 the log nuclear volume has been substituted for nuclear volume.

Examination of Table I, with the assumptions noted above in mind, will reveal that modal volumes of Class I are rare, and that the intermediate class occurs most frequently. It is perhaps significant that whereas 30% of the young adults have modal volumes of Class I, only about 5% of the tadpoles do. The number of kidneys in the tetraploid class (Class II) are most numerous through stages 7–10, 19–22 and in the young adults. In stage 7 through 10 and in the young adults 50% of the kidneys sampled give peak classes in the tetraploid range, while in stages 19–22 approximately 83% of the samples are in this range. It appears, therefore, that the greatest periods of mitotic activity occur within the stages indicated.

The logarithmic growth curve (Fig. 3) also serves to point out some of these relationships. The growth curve is a straight line drawn through points which represent the intermediate class described above. The scatter above and below the line is due to the occurrence of samples which give peak classes in the Class I and in the Class II range. Obviously, however, there are no marked increases or decreases in growth that can be correlated with age. All nuclear classes may be found in the kidneys of tadpoles which are of the same age, just as all nuclear classes may be found in the kidneys of tadpoles in the same stage (Table I). Nevertheless, a rough correlation can be made for stages 19–22. The animals in these stages are 45–50 days old. At 45–50 days the scatter is all above the line of growth indicating, perhaps, increased mitotic activity at this time.

A single bimodal curve was revealed in one of the individuals at stage 24 (Fig. 2). The two peaks correspond to the intermediate class and to Class II nuclei. Other instances of bimodality were suggested in the histograms. But in all cases except the one they were not revealed by either of the other graphing techniques, and are considered insignificant. With the one exception all histograms regardless of the technique were unimodal. However, when nuclear volumes are used in the histograms the curves show a marked asymmetry and are skewed to the right. This marked asymmetry disappears when the logs of nuclear volumes are used in place of the nuclear volume itself.

Discussion

The commonly accepted explanation for the appearance of the different nuclear classes in the frequency distribution curve is that for some nuclei growth in size is arrested at one or another of the growth stages, resulting in an accumulation of nuclei in the stage at which growth ceased. The tissue, as a result, carries accumulations of nuclei of differing sizes which are responsible for the peaks in the curve. Nuclei with volumes in the Class II range and greater, represent tetraploid

nuclei and the higher degrees of polyploidy, while those with volumes Class I–Class II represent interphasic sizes between successive mitoses. Hertwig (1939) in measuring nuclei from the early cleavage stages of mouse ova demonstrated that at each mitosis the nuclear volume was halved and he postulated that these volume changes could be correlated with similar changes in the genome. Alfert (1950), who studied cleavage stages in the mouse by photometric analysis of DNA content, reported doubling of DNA amounts in the interphase preceding mitosis. The significance of this observation derives from the fact that there is considerable evidence, obtained principally by the use of quantitative techniques, which suggests that DNA is either identical with the genic material or is at least closely associated with it. The same quantitative distributional patterns hypothesized by cytogeneticists for genic material can be directly applied to distributional patterns hypothesized for DNA. If this generalization is applied here, then a relationship must exist between nuclear volume and DNA content. Swift (1950) and Truong and Dornfeld (1955), using the photometric method for DNA determination in combination with caryometry on different animal tissues, showed that there is a definite relationship between DNA amount and nuclear size. Both volumes and DNA amounts fall into the ratio 1 : 2 : 4. In stable adult tissues (Swift, 1950, 1953; Pollister, Swift and Alfert, 1951) the DNA classes are clearly demarcated with little or no overlapping, while in actively dividing tissues there may be considerable overlapping because of the appearance of intermediate classes. The intermediate classes are due to gradual DNA synthesis during interphase. When the DNA reaches tetraploid level the nucleus enters prophase. After quantitative division of DNA at anaphase the diploid (Class I) amount of DNA is restored in the telophase nuclei. The cycle of DNA reduplication then repeats itself. Similarly, when labile tissues are studied by means of nuclear volume determinations, intermediate classes are revealed (Schreiber and Angeletti, 1940; Schreiber, 1949). In these cases the volume changes are interpreted as resulting from reduplicating phenomena in the genome.

In the kidneys of *R. sylvatica* used in this study there were no DNA determinations. Interpreting the results, however, on the basis of the above discussion it is possible to conclude that the data reveal the typical nuclear classes of a mitotic cycle, and a whole series of intermediate classes between Class I and Class II nuclei. The histograms (Figs. 1 and 2) show considerable overlapping. The overlapping is so marked that when the individuals are grouped and a combined histogram of all 63 kidneys is drawn, only one nuclear class is revealed. The modal value of this class falls approximately half way between Class I and Class II. It is generally believed that the increase in nuclear volume is rhythmic and that periods of rapid growth alternate with periods of relative inactivity. It is not possible from the results of this investigation to determine conclusively whether rhythmic growth as opposed to continuous growth is present here or not. For one thing, the degree of overlapping precludes any affirmative assumption concerning the presence of rhythmic growth. Not only are there no sharp demarcations between peaks suggesting discontinuity but there are also few positive correlations between the peak class and the stage and/or age of the animal. Secondly, the mere fact of the existence of a series of nuclear classes in a tissue does not imply that the details of individual nuclear growth are known, for normal frequency distributions may be

obtained not only from actively dividing tissues but from stable tissues as well (Bonner and Eden, 1956). However, the low incidence of Class I nuclei in the mesonephros of *R. sylvatica* may be due to a period of very rapid growth immediately following mitosis, so that Class I nuclei occur only transitionally. There is the possibility that the large percentage of nuclei in the intermediate class accumulate in this class due to a decrease in nuclear activity. Nuclei from this reservoir may be released gradually into the second growth period, at the end of which there is an accumulation of Class II nuclei. Schreiber and Angeletti (1940) found that in the mitotic cycle of hepatic cells of the carp there is a rhythmic increase and decrease in nuclear volume which can be correlated with the stage of development. In the kidneys of *Rana sylvatica* there is no such clearcut correlation. All nuclear classes may be present irrespective of the stage of development or of the age of the animal (Fig. 3). The developmental pattern in the kidney does not, therefore, reflect the details of nuclear growth. However, the percentage of Class II nuclei is greatest at stages 7–10, 19–22 and in the young adults. This suggests not only the possibility of three waves of mitotic activity but also a rhythmical growth pattern.

The nuclear size increase recorded here may not be associated with the duplicating process of the genome or with an increase in DNA. It has been discovered that in a given tissue, while DNA remains constant per nucleus, the protein content and the nuclear size show corresponding increases (Alfert, 1950; Biesele, 1944; Schrader and Leuchtenberger, 1950; Leuchtenberger and Schrader, 1951). Further, the chromosomal volume may increase by protein synthesis without an accompanying morphological change in nuclear size (Biesele, 1944). Also the nuclear volume may even be reduced as a result of water loss rather than by a change in the genome (Krantz, 1947). Furthermore, differences in nuclear size may have to do with nutritional differences that do not affect chromosomal size (Montgomery, 1910). Even in a normally dividing tissue there may be a size difference due to some factor other than that which stimulates cell activity. For instance, the age of the animal may affect cell or nuclear size quite independently of a tendency toward compensatory hypertrophy (Buchner and Glinos, 1950).

In view of these considerations a number of possibilities come to mind in regard to the increase in nuclear size observed here. This increase has been attributed to mitotic activity, or to an increase in chromosomal content. It is conceivable that some of this increase is due to imbibition or to an increase in the osmotic concentration consequent upon the synthetic activity of the chromosomes. Or synthetic activity in the interphase nucleus may alter the nuclear membrane, causing osmotic changes. This may effect an increase in nuclear volume which is not exactly paralleled by an increase in the volume of the chromosome. As a result nuclear volumes may vary to such an extent that there is considerable overlapping of volume classes in frequency curves. The increase in size of a nucleus may be partially due to mechanical pressure (Teir, 1949). In addition to the factors postulated above it is known that a morphological increase in size may be due to compensatory hypertrophy following such operations as unilateral nephrectomy (Sulkin, 1949) or partial hepatectomy (Sulkin, 1943), to hormonal agents like oestrone (Salvatore, 1950; Alfert and Bern, 1951; Schreiber, 1954), or to agents which stimulate chromosomal activity such as thiouracil (Roels, 1954), alloxan (Diermeier *et al.*, 1951) and colchicine (Bucher, 1951; Fankhauser, 1952). The administration of

any one of these agents may be followed by morphological changes in the nucleus, but need not necessarily be the direct cause of that change. Undoubtedly the size increase involves and is indicative of phenomena which are very complex due to the complexity of factors forming the cytoplasmic and nuclear ecology.

Some of the agents mentioned may not only be causative factors in increasing nuclear size but they may also induce polyploidy. Fankhauser (1952) by the use of colchicine induced endopolyploidy in embryos of the axolotl. Some of these embryos were originally diploid. It is generally known that polyploidy does occur in normal diploid animals. This condition, known as polysomaty or endopolyploidy, has been reported in the renal tubules of *Leptodactylus*, a South American frog, by Schreiber and Melucci (1949) and in the renal tubules of *Cyclorana*, the Australian desert frog by Dawson (1948). Polysomaty does not occur here in the kidneys of *R. sylvatica*. Twenty-six of the kidneys give modal volumes belonging to Class II. Conceivably, some of the nuclei in this peak class might be true polyploids with twice the diploid number of chromosomes rather than interphasic cells approaching a proliferative stage. There is no way of determining this, however, by the method used here. The histograms gave no peak classes at higher than the hypothetical tetraploid level. Possibly, some of the very large nuclei are octoploids. If so they are so rare that there is no great accumulation at this level and therefore the higher peaks are not obtained. In tissues showing a relatively high incidence of polyploid cells the frequency increases with age (Swartz, 1956). Polyploid cells may be absent then in the tadpole kidneys and in young adults and may appear only in older animals. However, it is now a well known fact (*cf.* Fankhauser, 1945) that polyploidy does occur in tadpole tissues. It can only be concluded, therefore, that there are few if any polyploid cells in the mesonephroi of these *R. sylvatica*. This is consistent with the general opinion that the incidence of polyploidy in kidney tissues is relatively rare, even though it is known to occur in those instances cited above.

The concept of endopolyploidy, or any concept of variation in chromosome number, renders untenable the hypothesis that for a given species the chromosome number is the same for all of the somatic cells. The "constancy hypothesis" has, therefore, been revised on the assumption that DNA is somehow related to the genic material. The constancy of DNA per chromosome set, first proposed by Boivin, Vendrely, and Vendrely (1948), and by Ris and Mirsky (1949), is supported by the work of Pollister and his school (*cf.* Pollister, Swift and Alfert, 1951). As pointed out above, increased metabolic activity in the chromosomes as shown by DNA synthesis is accompanied by an increase in nuclear size. If this increase is always proportionate to DNA increase then a constant relationship should exist between DNA content per chromosome set and nuclear size. Obviously, such a constant relationship does not exist. The phenomenon of variation in nuclear size may very well be a secondary event which may or may not be associated with DNA content. In terms of "constancy," assumptions concerning chromosome number and probable DNA content cannot always be made from nuclear volume data. There is also a certain amount of evidence which calls into question the constancy of DNA per chromosome set. Roels (1954) working with rat thyroid and Diermeier *et al.* (1951) with the liver of alloxan diabetic rats have presented evidence to indicate that the DNA content of a cell may vary with its degree of functional activity. Pasteels and Lison (1950) concluded that the DNA content of certain rat tissues is lower than the diploid value because of chromatin diminution. Though this particular evi-

dence has been questioned (Alfert and Swift, 1953), chromatin diminution is known to occur in *Ascaris* (Boveri, 1904) and in some other forms (see the brief review of Tyler, 1955.) Also, Marshak and Marshak (1955) discuss negative DNA reactions in the *Arbacia* egg. Their evidence, however, has also been questioned (*cf.* Burgos, 1955; Marshak and Marshak, 1956). The exceptions to DNA constancy still raise a question in regard to the chemical nature of the gene, and they must be kept in mind when interpreting data having to do with nuclear volumes.

The occurrence of mitotic activity in the mesonephroi herein investigated indicates that during metamorphosis the kidney has some cells that are undifferentiated. At least physiologic inactivity in terms of renal function can be postulated for the proliferating cells. Some of the non-dividing cells, however, must be already manifesting renal activity. If so then the nuclear sizes may be indicative of a differentiating process. The fact that nuclear size and characteristic cell structure are intimately related is most dramatically demonstrated in insects. For example, in certain honeybee tissues (Merriam and Ris, 1954) the nuclear volume not only increases with age but there is a direct correlation between the nuclear size and secretory activity. Of equal importance is the fact that in certain mammalian tissues such as rat liver the increase in numbers of large nuclei closely parallels the histological and functional development of the organ (Sulkin, 1943; McKellar, 1949). A somewhat similar relationship has been demonstrated for a species of the slime mold, *Dictyostelium* (Bonner, 1957). In this mold, the stage of development and the early stages of differentiation are reflected in both the cell size and in the nuclear size which is characteristic of the particular stage. Further, the most active cells of this slime mold are located at the anterior end of a migrating sausage-shaped mass. The active cells are also the larger and are, according to Bonner, responsible for important morphogenetic effects which end in the formation of the fruiting body. Another parallel between cell size and differentiating activity may be seen in the developing sea urchin egg. It is well known that the micromeres formed during the cleavage stages of the sea urchin egg play an important role in morphogenesis in that they induce vegetal differentiation. McMaster (1955), working with *Lytechinus*, has shown by photometric determinations of DNA amounts in the cleavage cells that the lowest DNA amounts are in the micromeres. It is possible that the differentiating effects of these cells may be due to the lower DNA values. Once again, therefore, the point to be made here is that cell size is associated with a differentiating process. Progressive differentiation may also be associated with quantitative differences in DNA amounts in amphibians. Moore (1952), in working with haploid and diploid tissues of *Rana pipiens* embryos, found that the range of DNA values in the forebrain was greater in the 7-day embryos than in the 11-day embryos. She found no correlation between mitotic activity and the amount of DNA. In explaining this she discusses a possible correspondence between DNA amount and differentiation. DNA may decrease with age and with the maturation of the tissue until it reaches some more or less constant value. If this is so then nuclear volumes may also be smaller in adults than in embryos. Perhaps increased differential activity accounts for the larger percentage of Class I nuclei recorded (Table I) here for the young adults of *Rana sylvatica*.

At histological maturation differentiation may be influenced by polysomaty. Polyploid cells at the time of differentiation may return to a lower value by some process such as reduction mitosis (Huskins, 1948). The genic segregation which

accompanies the reductional division may produce cells whose differentiating potential is quite different. This interpretation puts stress on ploidy as a causative factor in differentiation. In considering polyploid organisms it is known that generally they differentiate normally and effects of ploidy, such as larger nuclear or cell size, are considered secondary. Mather (1948) has insisted that duplication of the chromosome number is not the cause of differentiation. The fate of the cell, he concluded, is dependent not so much on the nucleus which in most cases is diploid in sexually reproducing animals or plants, but on a cytoplasm which is inherited from the past. The action of the nucleus is effective only because the cytoplasm has changed at each step along the way. At each division the nuclei are quantitatively and qualitatively alike but each one inherits a portion of cytoplasm, one portion of which cannot be equated with the other. If polyploidy occurs it is probably in response to the cytoplasm.

In conclusion, the difficulties of interpreting nuclear size relationships are many and varied. Not only are the techniques used for such studies possessed of their own difficulties but nuclear size itself may be altered by a number of conditions. Size changes may be physiological, mitotic, or due to different degrees of heteroploidy. If genic changes are involved then these changes may be correlated with alterations in DNA amounts. The results strongly suggest that DNA is the genic material. But the fact of the matter remains that the gene is a biological concept, an abstraction, known only in its effects. The exact relationship between the gene and DNA is still unknown. A nuclear size change, itself, may be a phenomenon occurring coincidentally with differentiation, as if there were two progressions, one of which functions as the cause or the effect of the other. Or instead, all the events involved may converge and commingle as an expression of a single phenomenon reaching peak expression in the differentiated living cell. Increases or decreases in nuclear size are morphological and physiological events which take part in this convergence.

Summary

1. The mesonephroi from 63 *Rana sylvatica* tadpoles and young adults were studied in sectioned material by means of a caryometric method for determination of nuclear volumes. Frequency histograms drawn from the data reveal three peak classes which, when arranged in series, give the progression, 1 : 1.5 : 2. These results are interpreted as being due to an interphasic growth preceding mitosis.

2. The results are discussed in terms of morphological increase and decrease in nuclear size, increase and decrease of DNA values, polyploidy, the "constancy" hypothesis, physiological activity and histological differentiation.

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THE ROLE OF ADSORPTION AND MOLECULAR MORPHOLOGY IN OLFACTION: THE CALCULATION OF OLFACTORY THRESHOLDS

J. T. DAVIES AND F. H. TAYLOR[1]

Dept. of Chemical Engineering, Pembroke Street, Cambridge, England, and Dept. of Chemistry, Imperial College, South Kensington, London, S. W. 7, England

The surface of the olfactory nerve cell is folded into a number of delicate, hair-like protoplasmic filaments (Engström and Bloom, 1953; Bloom and Engström, 1952) which have long been considered to be the ultimate sensory processes of the receptor cells. Admirable photographs showing these hairs, a discussion of their possible functions and a description of the anatomy of the entire olfactory epithelium of various vertebrates are given by Le Gros Clark (1957) and by Allison (1953).

The filaments serve to give the olfactory receptors a large surface area. That this enlarged surface readily adsorbs odorants was shown by Moncrieff (1954, 1955) who made direct measurements, using a sheep's head, and found that odorants are adsorbed strongly and rapidly on the olfactory epithelium and that the process is reversible.

Neither the structure nor the exact composition of the human olfactory cell membrane is known. Hopkins (1926) examined the olfactory filaments of the frog which he found to be extensions of the olfactory cell membrane; he noted that the hairs reduce osmic acid and are disrupted by organic lipoid solvents, the proximal parts only remaining intact. The human olfactory membrane will probably resemble that of the frog and, like other nerve cells, will consist of a few layers of oriented lipid and protein molecules. This membrane is continually bathed by mucus (effectively saline) at a pH of about 7.2.

Davies and Taylor (1954) used the erythrocyte membrane as a model for that of the olfactory nerve cell, and showed that a large number of odorous substances act as accelerators of haemolysis by saponin. Moreover, for these substances, the logarithms of the olfactory thresholds (for man) are directly proportional to the logarithms of the haemolytic accelerating powers. This is shown in Figure 1. Some of these compounds which act as haemolytic accelerators have also been shown to cause a leakage of potassium ions across the red cell membrane (Davson and Danielli, 1938). This correlation lends support to theories of olfaction such as those of Ehrensvärd (1942) and Davies (1953a, 1953b) which postulate that odorant molecules must first adsorb onto the plasma membrane of the olfactory cell. Ehrensvärd's theory that the potential changes due to adsorption at this interface initiate the nerve impulses ascribes more importance to the specific polar groups of the odorant molecules than does the present work. Further experiments on potentials on the lines of those of Ehrensvärd and Cheesman (1941) would be of interest in this connection.

[1] Beit Memorial Fellow for Medical Research.

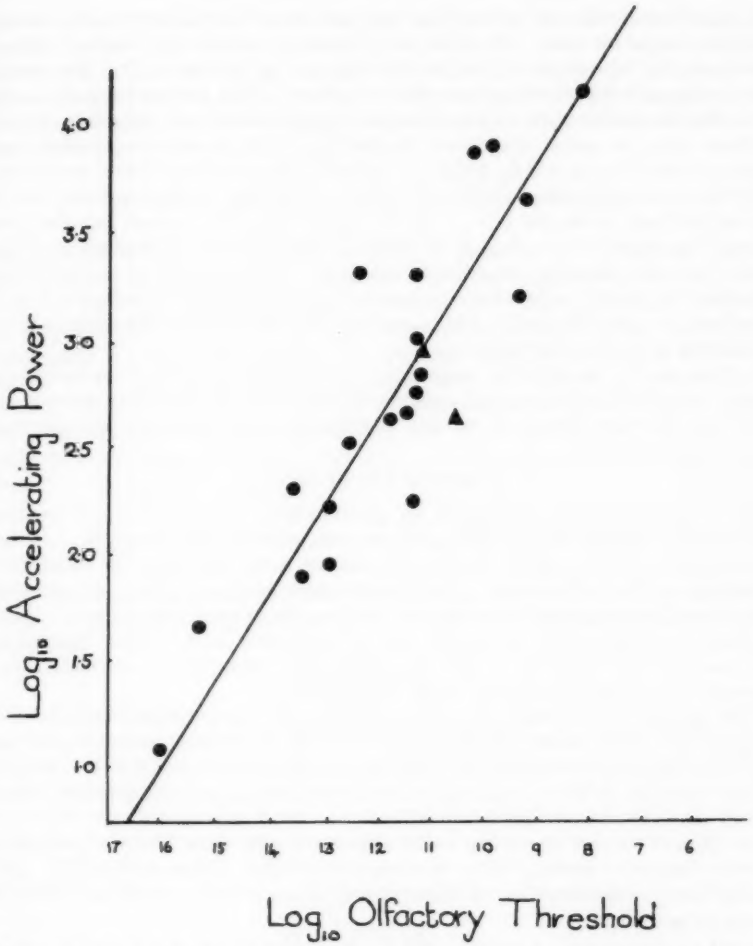

FIGURE 1. Plot of the logarithm of the haemolytic accelerating power against the logarithm of the olfactory threshold (in molecules/cc.). The equation of the line which is drawn in can be deduced from equation (4) if certain approximations are made (Davies and Taylor, 1957).

The present work, following the theory of Davies, assumes that the odorant molecules can simply dislocate the olfactory cell membrane, permitting an exchange of sodium and potassium ions across it. This exchange of ions initiates the nervous impulse as in the general theory of Hodgkin and Katz (1949).

Recently, Rideal and Taylor (1958) have shown that the logarithm of the haemolytic accelerating power of a substance is directly proportional to its free energy of adsorption at the oil-water interface; this means that the effectiveness of compounds as haemolytic accelerators depends only on the number of mole-

cules adsorbed on the red cell surface and not, in addition, upon such factors as molecular shape or size. In olfaction, however, additional factors must be important since the slope of the line in Figure 1 is not unity and also because there is some scatter of the points about the line. This scatter may, in part, be due to the roughness with which olfactory measurements can be made, but it is also likely that the sizes, shapes and flexibilities of the penetrating molecules are important (see Davies, 1953a, 1953b). Indeed, Timmermans (1954) and Mullins (1955) have recently emphasised this point. Further, among isomers, the one with the bulkiest molecule (*i.e.*, with the most branched chains) has the lowest olfactory threshold even though it may be less strongly adsorbed at a fatty surface than the corresponding linear molecule. This applies to *n*-amyl alcohol (threshold 6.8×10^{12} molecules/cc.) and to isoamyl alcohol (threshold 6.8×10^{11} molecules/cc.) (von Skramlik, 1948) and also to the isomeric decanols; it is true for *Phormia* as well as for humans.

In this paper, the relative importance of adsorption and of molecular morphology in olfaction is assessed quantitatively, and an equation derived from which the olfactory threshold of any particular compound may be calculated.

Theoretical Section

For mathematical convenience, we assume that an olfactory cell surface is divided into n small, non-specific areas or sites, each of area α sq. cm. In order to stimulate a cell, a critical number of odorant molecules must be concentrated on one site in the cell surface. For the strongest odorant, which is assumed to be β ionone, perhaps only one molecule need occupy one of these areas. Weaker odorants, however, such as acetic acid or methanol have a less "dislocating" effect on the cell membrane and so p molecules must be concentrated simultaneously on one of the sites to cause a response.

The quantity $1/p$, then, is a measure of the "puncturing" ability of the odorant, *i.e.*, of the effectiveness of the molecule in causing the necessary ionic leakage across the cell membrane. For the strongest odorants $1/p = 1$ whilst for weaker odorants $1/p < 1$, and for water which is continually bathing the cell without causing any stimulus, $1/p = 0$.

To adsorb on the sites, the molecules must pass from the air through the aqueous (mucous) phase. This process is reversible (Moncrieff, 1955) and at equilibrium the distribution of molecules is given by (1) which is a simplified Langmuir isotherm.

$$\frac{x}{c\,d} = K_{L/A}. \tag{1}$$

Here x is the average membrane concentration of odorant molecules/sq. cm., c is the average concentration of molecules/cc. in the air, and d is the surface thickness (about 10 Å). $K_{L/A}$ is the adsorption constant for molecules passing from air to the lipid-water interface. The number of sites, N, per nerve cell containing p molecules where p is greater than the average number αx is given by Poisson's equation:

$$N = \frac{n\,e^{-\alpha x}(\alpha x)^p}{p!}, \tag{2}$$

where n is the total number of sites/cell.

At the olfactory threshold, only one site with the p adsorbed molecules will be required to stimulate the cell minimally, so that N is unity and c is the olfactory threshold. By combining equations (1) and (2) we can eliminate x and obtain a relation between $K_{L/A}$, p and the olfactory threshold. The relation in its final form is given by (3) and has been derived in full elsewhere (Davies and Taylor, 1957).

$$\log \text{O.T.} + \log K_{L/A} = \frac{-\log n}{p} + \frac{\log p!}{p} - \log \alpha d. \tag{3}$$

Calculation shows that for the weakest odorants possible, p lies between 15 and 30 and an arbitrary value of 24 is taken. For these compounds the quantity (log O.T. + log $K_{L/A}$) tends towards a value of 22. The compound with the lowest recorded olfactory threshold (β ionone; as listed by von Skramlik, 1948, and confirmed by the measurements of Neuhaus, 1953b) is assumed to have a p value of 1. If any more powerful odorant were discovered and assigned a p value of unity, the numerical values of the thresholds predicted would change slightly, but their relative values would not.

Application of these boundary conditions enables values of the constant log n and log αd to be found; equation (3) then takes the form:

$$\log \text{O.T.} + \log K_{L/A} = \frac{-4.64}{p} + \frac{\log p!}{p} + 21.19. \tag{4}$$

Using this equation we can calculate the olfactory threshold of any substance, knowing its adsorption constant between air and the lipid-water interface and its value of p.

Methods

The adsorption constants for molecules going from water to the oil-water interface have been determined from measurements of the lowering of the interfacial tension at the petroleum ether-water interface (Haydon and Taylor, 1959). Non-polar compounds (*e.g.*, hydrocarbons) will dissolve in the lipophilic interior of the membrane, and so for these compounds, distribution constants for molecules passing from water to the petroleum ether (bulk) phase have been used. Since we are dealing with a fatty membrane, the adsorption at the membrane/water interface will be approximately equal to that at the oil-water interface.

$$K_{O/W} = K_{L/W}.$$

In fact $K_{L/W}$ will be slightly less than $K_{O/W}$ by a factor depending on the dielectric constant at the membrane surface. The distribution constant for molecules going from air to the aqueous phase has been found from the ratio of the solubility of the substance in water at 20° C. to its vapour pressure at the same temperature, or, in some instances, from the measurements of partial vapour pressures of aqueous solutions of the substances recorded in the literature.

From $K_{L/W}$ (= $K_{O/W}$) and $K_{W/A}$, the term $K_{L/A}$ (= $K_{O/A}$) required in equation (4) may be obtained directly since

$$K_{L/A} = K_{L/W} \cdot K_{W/A}.$$

Values of log $K_{O/A}$ are listed in Table I.

TABLE I

Compound	$\log_{10} K_{O/W}$	$\log_{10} K_{W/A}$	$\log_{10} K_{O/A}$	Cross-sectional areas Å²	
				a	b
Methanol*	1.235	4.245	5.48	20.0	12.4
Ethanol*	1.80	3.99	5.78	26.7	15.4
Propanol*	2.41	3.81	6.22	32.6	18.3
Butanol	2.85	3.69	6.54	38.0	19.2
Pentanol	3.71	3.42	7.13	43.0	20.0
Hexanol	4.39	3.18	7.57	47.8	20.0
Heptanol	4.82	2.83	7.65	52.3	20.0
Octanol	5.30	3.04	8.34	56.0	20.0
Decanol	6.94	2.04	8.98	64.6	20.0
β ionone	5.79	2.56	8.35	69.7	57.6
Piperonal	3.41	3.48	6.89	47.3	41.4
Menthol	4.505	2.015	6.52	62.8	44.8
Skatol	4.76	3.84	8.60	53.5	42.5
Xylol musk	4.58	3.96	8.54	74.0	56.2
Isoamyl alcohol	3.71	3.50	7.21	43.0	26.0
Isobutanol	2.26	3.81	6.07	38.0	24.0
Camphor	3.335	2.64	5.98	59.7	43.6
Phenol	3.04	4.66	7.70	37.9	24.1
Isoamyl acetate	3.63	1.82	5.45	52.6	30.0
Nitrobenzene	3.85	8.14	6.99	42.2	24.1
Coumarin	2.83	4.48	7.31	48.0	41.3
Pyridine*	3.25	4.78	8.03	36.3	24.0
Cycloheptadecyl lactone	5.33	3.42	8.75	89.0	50.0
Glycerol*	1.93	4.00	5.93	36.5	36.0
Cyclohexanol	3.92	4.05	7.97	45.5	27.0
Water	—	—	—	14.0	8.1
Naphthalene**	4.67	1.82	6.49	48.5	42.0
Benzene**	3.09	0.75	3.84	36.1	24.0
Cyclohexane**	3.56	−0.35	3.21	43.6	27.0
Ethane**	1.23	−0.66	0.66	24.4	15.4
n-butane**	2.40	−0.60	1.80	36.1	20.0
n-pentane**	2.97	−0.66	2.31	41.2	20.0
n-heptane**	4.15	−0.59	3.56	50.6	20.0
n-nonane**	5.33	−0.76	4.57	59.3	20.0
n-undecane**	6.52	−0.74	5.78	67.3	20.0
n-butyric acid*	3.19	6.17	9.36	38.7	20.0
n-valeric acid	3.78	5.04	8.82	43.8	20.0
Caproic acid	4.38	4.85	9.23	84.5	20.0
Oenanthic acid	4.97	4.55	9.52	53.0	20.0

Data needed to calculate olfactory thresholds for a number of odorants.

* $K_{W/A}$ calculated from published data on the partial vapour pressures of aqueous solutions of the substances.

** $K_{O/W}$ for non-polar compounds refers to passage from water to bulk of the oil phase.

The cross-sectional areas in column a have been calculated from the molecular volumes assuming the molecules to be spheres. The molecular volumes have been obtained by assuming the additivity of atomic volumes using data given in Partington (1951).

Areas in column b have been measured from models.

Results and Discussion

(a) *The calculation of olfactory thresholds*

Since $1/p$ is a measure of the ability of an odorant molecule to "puncture" or dislocate the membrane temporarily, it is expected to be a function of molecular shape and size. These quantities are difficult to define, however, and for present

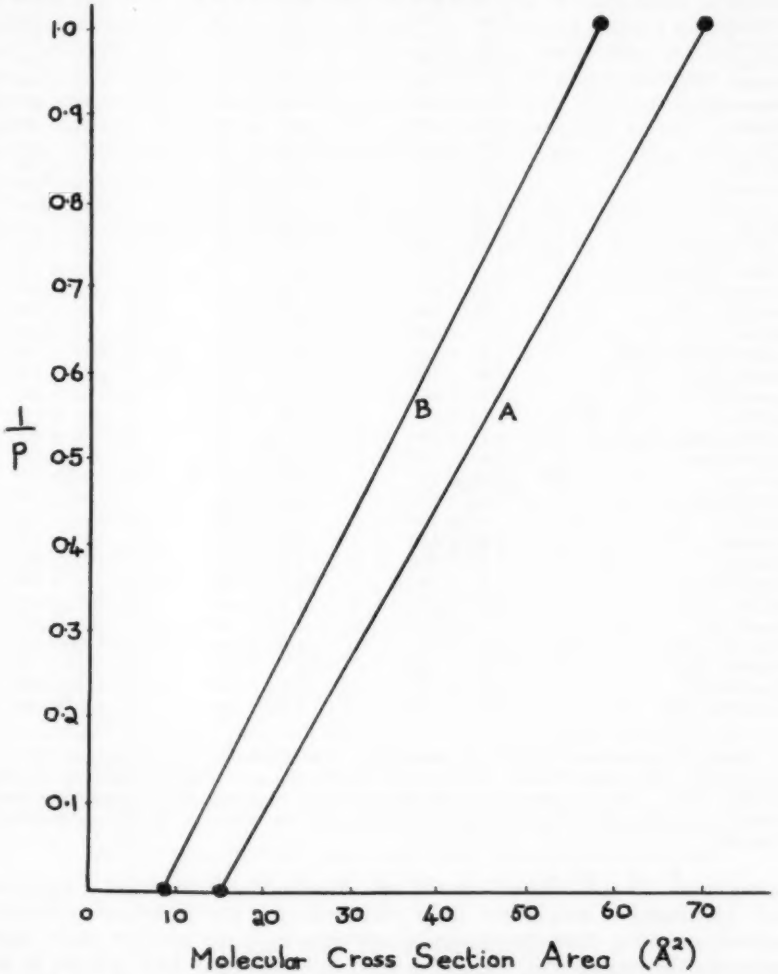

FIGURE 2. The relationships which are assumed to hold between $1/p$ and the molecular cross-sectional areas in the evaluation of $1/p$, when (A) cross-sectional areas derived from molecular volumes are employed (molecules assumed to be spheres). (B) Measurements on models have been employed (molecules uncoiled and orientated in the membrane).

TABLE II

Compound	Olfactory thresholds in molecules/cc.		
	Observed	a	b
Methanol	1.10×10^{16}	8.13×10^{15}	1.15×10^{16}
Ethanol	2.44×10^{15}	6.61×10^{14}	2.04×10^{15}
Propanol	5.00×10^{13}	5.13×10^{13}	3.00×10^{14}
Butanol	8.20×10^{12}	7.94×10^{12}	1.12×10^{14}
Pentanol	6.80×10^{12}	9.78×10^{11}	2.14×10^{13}
Hexanol	6.72×10^{12}	1.32×10^{11}	7.76×10^{12}
Heptanol	9.00×10^{11}	5.25×10^{10}	6.46×10^{12}
Octanol	3.00×10^{10}	4.27×10^{9}	1.32×10^{12}
Decanol	3.63×10^{11}	1.78×10^{8}	3.02×10^{11}
β ionone	1.60×10^{8}	1.60×10^{8}	1.60×10^{8}
Piperonal	2.00×10^{11}	7.08×10^{11}	3.39×10^{11}
Menthol	2.00×10^{11}	8.32×10^{11}	3.31×10^{11}
Skatol	1.80×10^{9}	4.07×10^{9}	4.47×10^{9}
Xylol musk	2.10×10^{9}	4.47×10^{7}	1.58×10^{8}
Isoamyl alcohol	6.80×10^{11}	8.13×10^{11}	3.63×10^{12}
Isobutanol	8.20×10^{12}	2.34×10^{12}	8.13×10^{13}
Camphor	5.00×10^{12}	4.90×10^{11}	1.45×10^{12}
Phenol	7.90×10^{12}	5.37×10^{11}	1.82×10^{12}
Isoamyl acetate	1.82×10^{14}	6.92×10^{12}	8.51×10^{13}
Nitrobenzene	2.00×10^{11}	1.32×10^{12}	9.55×10^{12}
Coumarin	2.10×10^{11}	2.40×10^{11}	1.32×10^{11}
Pyridine	3.10×10^{11}	3.63×10^{11}	8.91×10^{11}
Cycloheptadecyl lactone	1.75×10^{10}	2.76×10^{8}	5.50×10^{8}
Naphthalene	2.60×10^{12}	1.45×10^{12}	3.09×10^{13}
Benzene	4.00×10^{13}	5.75×10^{15}	1.38×10^{16}
Cyclohexane	1.82×10^{15}	6.46×10^{15}	2.88×10^{16}
Ethane	1.30×10^{19}	1.70×10^{20}	6.31×10^{19}
n-butane	7.11×10^{16}	6.31×10^{17}	4.57×10^{18}
n-pentane	2.73×10^{16}	7.59×10^{16}	1.41×10^{18}
n-heptane	1.32×10^{16}	8.51×10^{14}	7.94×10^{16}
n-nonane	4.87×10^{15}	1.32×10^{13}	7.76×10^{15}
n-undecane	1.35×10^{15}	1.15×10^{11}	4.79×10^{14}
n-butyric acid	1.4×10^{11}	1.05×10^{10}	1.26×10^{11}
n-valeric acid	1.2×10^{11}	1.55×10^{10}	4.37×10^{11}
Caproic acid	1.2×10^{12}	2.63×10^{9}	1.70×10^{11}
Oenanthic acid	1.35×10^{13}	5.13×10^{9}	8.71×10^{10}

Observed and calculated olfactory thresholds. The observed values have been taken from those listed in the publications of von Skramlik (1948), Backman (1917), Morimura (1934) and Mullins (1955). The calculated values listed in column a have been obtained assuming that the molecules are spherical. Those in column b assume that the odorant molecules are uncoiled and orientated.

purposes the cross-sectional areas of the molecules are employed. These have been calculated in two ways: those obtained from the molecular volumes are derived assuming that the molecules are spherical; for straight chain organic molecules this means that the chains must be coiled up. This is likely to be so when the molecules are in water but is unlikely in a fatty membrane. In measurements taken from models, therefore, it is assumed that the molecules adsorbed in the membrane are orientated with the hydrocarbon chains completely uncoiled;

in a homologous series the cross-sectional area is thus constant for the higher members.

If it is assumed that the dislocating power $1/p$ varies linearly with the molecular cross-sectional area, then values of $1/p$ can be calculated, since for β ionone $1/p = 1$ and for water $1/p = 0$ (Fig. 2). Values of the areas used for this purpose are listed in Table I.

From $1/p$ and log $K_{O/A}$ olfactory thresholds can be calculated using (4); values thus obtained are included in Table II. In Figures 3 and 4 the observed and calculated values are compared, assuming respectively that the molecules are spherical and that they are unfolded and orientated. It is seen that there is good agreement between calculated and observed values. This encourages us to believe that the "puncturing theory" of olfaction is essentially correct and that, to cause a stimulus, more small molecules must be packed into a given area of membrane surface than large ones.

In general, and for the aliphatic hydrocarbons and alcohols in particular, the observed thresholds tally much better with those calculated assuming that the

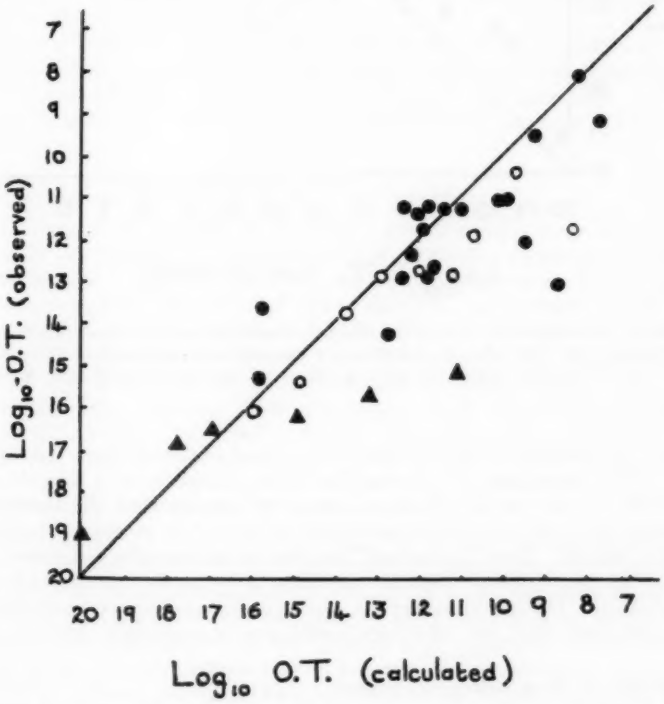

FIGURE 3. A comparison of observed olfactory thresholds and those calculated from equation (4) assuming that the molecules are spherical. ○ Values for normal alcohols; ▲ values for normal paraffins; ● other compounds.

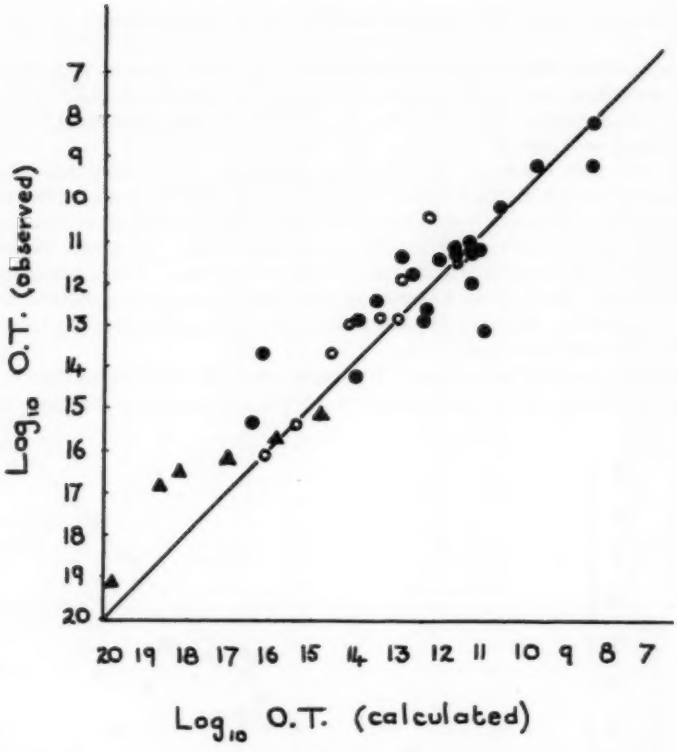

FIGURE 4. A comparison of observed olfactory thresholds and those calculated from equation (4) assuming that the odorant molecules are uncoiled and orientated in the olfactory cell membrane. ○ Values for normal alcohols; ▲ values for normal hydrocarbons; ● other compounds.

molecules are unfolded (Fig. 4) than with those obtained from the molecular volumes. The constancy of $1/p$ for the higher members of a homologous aliphatic series, as can be seen from equation (4), means that the thresholds for these compounds will decrease less rapidly than if $1/p$ increased continuously with chain length. This "tailing off" for the higher members of a series is observed for the thresholds of aliphatic alcohols, acids, hydrocarbons and with the chloroparaffins. The better agreement shown in Figure 4 (compared with Fig. 3) confirms the idea that the olfactory membrane is essentially fatty in nature.

(*b*) *Conditions at the olfactory cell surface*

In obtaining equation (4) from equation (3) by the use of experimental boundary conditions, values for log n (where n is the number of "sites" per cell) and α the area of one "site" are obtained. Log n is 4.64 which means that there

are 4.5×10^4 "sites" per cell and α has the value of 64 Å^2 if the depth of the surface phase is taken to be 10 Å.

The active adsorbing surface area of each cell then comes to $n\alpha$ or 3×10^{-10} sq. cm. This is much smaller than that which can be calculated from the results of Bloom and Engström (1952) (3×10^{-6} sq. cm.) or those of Le Gros Clark and Warwick (1946) (ca. 10^{-8} sq. cm.). This could either indicate that the active "sites" do not occupy the entire cell surface or that one of the boundary conditions used in deriving (4) is wrong. If, for instance, there is a stronger odorant than β ionone (such that log O.T. < 8.2), then the surface area predicted would be larger.

(c) Prediction of olfactory thresholds

If the values of $K_{O/W}$ and $K_{W/A}$ are determined experimentally for any substance, and if its molecular dimensions are known approximately, then it is possible by use of equation (4) to predict its olfactory threshold. Thus for cyclohexanol log $K_{O/W}$ is 3.92, log $K_{W/A}$ is 4.05 and from its molecular cross-sectional area, $1/p$ is 0.37 (using Figure 2). From equation (4) we now predict a threshold of 5×10^{11} molecules/cc.

Glycerol will have a value of about 0.32 for $1/p$ and the values of log $K_{O/W}$ and log $K_{W/A}$ are 1.93 and 4.0, respectively. The predicted threshold is then about 10^{14} molecules/cc. However, the saturation concentration in the air is much less than this (ca. 10^{13} molecules/cc.) at room temperature so that it cannot be detected.

(d) The effect of temperature on the olfactory threshold

The molecular dimensions of an odorant and therefore, presumably, $1/p$ will not alter as the temperature is raised and so the right hand side of (4) will be constant for any odorant. However, $K_{L/W}(K_{O/W})$ decreases for organic compounds as the temperature is raised; the value of $K_{W/A}$ also decreases numerically (thus at 20° C. $K_{W/A}$ for *n*-hexanol is 1.54×10^3 whilst at 40° C. it is 2.29×10^2).

For most compounds, therefore, $K_{O/A}$ decreases as the temperature is increased. This means that as the temperature is raised the olfactory threshold should attain higher values. This is in accord with the findings of Morimura (1934) who reported that small increases in temperature raise the threshold appreciably.

(e) Olfaction and chemoreception in insects

The receptors of olfaction in *Phormia* (the blowfly) are situated on the antennae and labellae whilst those of the contact chemical sense are on the tarsi. Dethier and his co-workers have been able to measure rejection concentrations for one sense by removal of the receptors of the other: thus by using antennectomized and labellectomized insects Dethier and Chadwick (1948, 1950) were able to measure rejection concentrations for tarsal chemoreception uncomplicated by olfaction. These concentrations are plotted in Figure 5 against log $K_{O/W}$ and there is seen to be a linear relationship between the two quantities, the equation of the line being

$$\log M = 1.17 \log K_{O/W} + 2.83.$$

Here M is the threshold expressed as the molar concentration of test substance *in water* rejected by 50% of the flies. The slope of the line is very near to that expected for adsorption from solution on to a pure lipid membrane (1.0).

The olfactory rejection concentrations, however, (given by the molar concentrations in *the air* rejected by 50% of the insects) when plotted against log $K_{O/A}$,

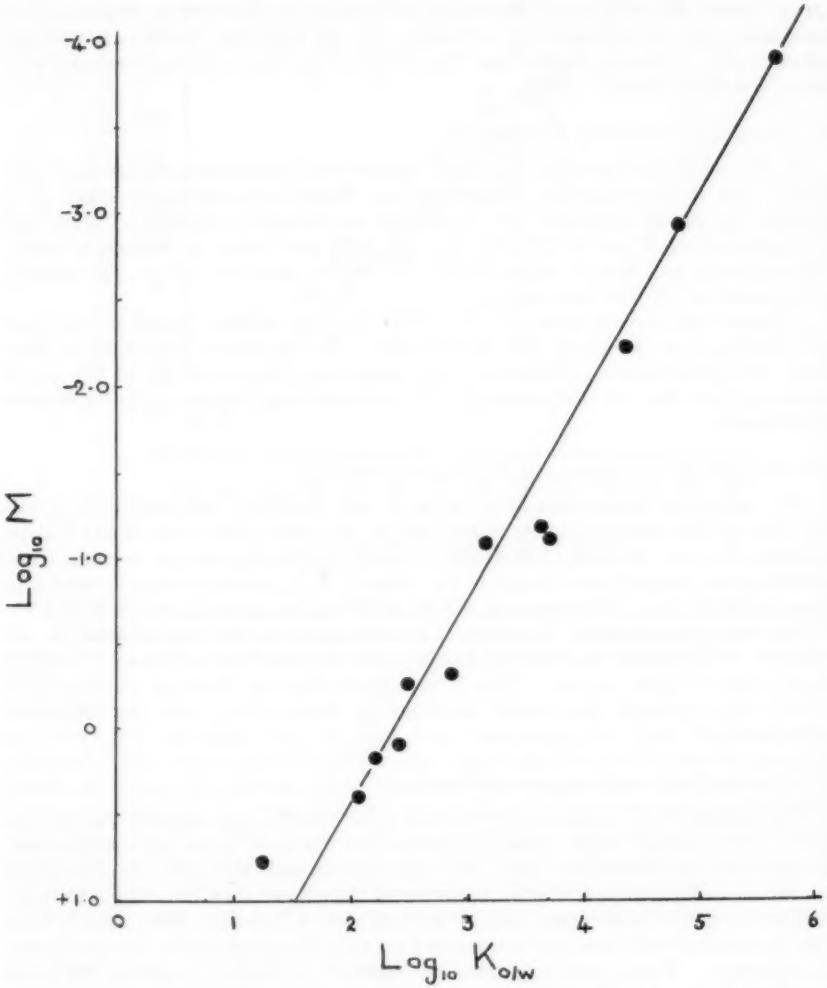

FIGURE 5. Plot of the logarithm of the molar concentration in water rejected by 50% of a number of blowflies against $\log_{10} K_{O/W}$. The concentrations are taken from the publications of Dethier and Chadwick (1948, 1950).

show a levelling off for the higher members of a homologous series (Fig. 6). This confirms that a shape factor in addition to an adsorption factor is significant in olfaction. Unfortunately, results are available only for aliphatic alcohols and aldehydes for *Phormia* (Dethier and Yost, 1952; Dethier, 1954).

Examination of the literature shows that there are many examples of the interaction of small molecules with biological membranes where adsorption is all-important (as with the chemoreception above or in haemolytic acceleration) and others where the effect is dependent on molecular shape or polarity in addition to the membrane concentration. Thus in the action of compounds on *B. typhosus*

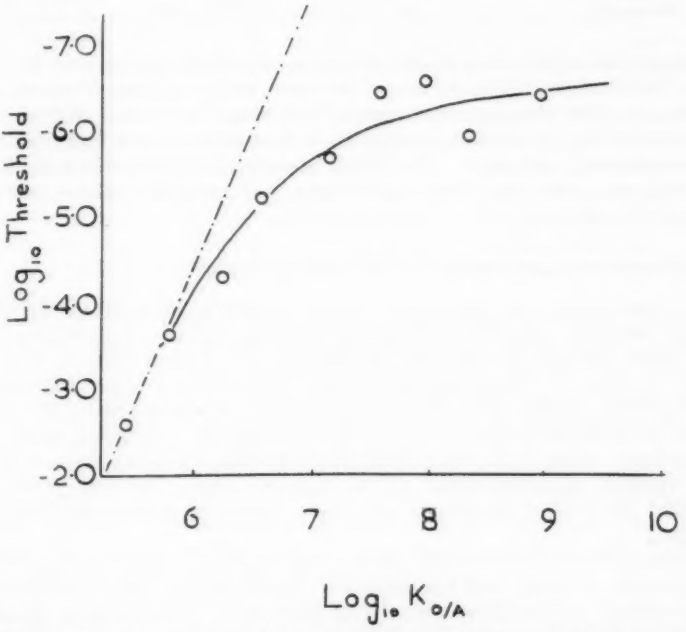

FIGURE 6. Plot of the logarithm of the olfactory rejection concentration for blowflies against $\log_{10} K_{O/W}$. The concentrations are taken from Dethier and Yost (1952). ·—·—·—·—·, line with slope expected if $1/p$ were to increase regularly with chain length for alcohols.

the effect is proportional to log $K_{O/W}$ (Ferguson, 1939) but the action of similar substances on chloroplast lecithinase systems (Kates, 1957) involves a shape or polarity factor in addition.

(f) Olfaction in dogs

Several workers have recently determined olfactory thresholds for dogs (Krushinski, Chuvaev and Vollkind, 1946; Uchida, 1951; Neuhaus, 1953a, 1953b; Ashton, Eayrs and Molton, 1957). The carefully conducted experiments of Ashton, Eayrs and Molton, using solutions of fatty acids, yield thresholds in

terms of gm. mols./liters of *aqueous* solution. From the values of $K_{W/A}$ given in Table I, these can be converted into molecules/cc. in air, assuming that equilibrium conditions prevailed during the experiments. The results for four acids are given below.

Thresholds

Acid	Log molar conc. in water	Molecules/cc. in air	
		Calculated	From Neuhaus (1953a, 1953b)
Butyric	−4.38	1.73×10^{10}	9×10^{3}
Valeric	−3.29	2.82×10^{12}	3.5×10^{4}
Caproic	−3.56	2.34×10^{12}	4.0×10^{4}
Oenanthic	−3.87	2.29×10^{12}	—

The thresholds are 10^7 times higher than those obtained more directly by Neuhaus and by Buytendijk (1921) and are of the same order as those observed for man. This means either that the experiments of Ashton, Eayrs and Molton were not carried out under equilibrium conditions or that dogs are less sensitive to odours than is commonly supposed. The levelling-off of the thresholds with the higher acids suggests, once more, that adsorption *and* molecular shape and size are important in olfaction.

(g) *Qualitative aspects of odour and the intensity factor*

The quantitative success of our theory supports, we believe, the idea that adsorbed odorant molecules initiate the nervous impulse by causing localized permeability changes in the cell membrane. It is clear that to be a strong odorant a substance must possess a large value of $K_{L/A}$ and a low value of p. Few molecules exhibit both characteristics since, if p is decreased by the introduction of branched chains, $K_{L/A}$ decreases markedly. Artificial musk (trinitro tertiary-butyl xylene) possesses good olfactory characteristics because the molecule is strongly adsorbed whilst $1/p$ is relatively high. Mullins (1955) has also concluded that rigid molecules are more effective stimulants than flexible molecules.

Mullins (1955) has recently shown that carefully purified *n*-paraffins definitely possess an odour and has measured thresholds for these compounds. The present theory, unlike those postulating interaction between polar groups, predicts this and the threshold values that we have calculated agree well with those observed by Mullins (Figs. 3 and 4).

It should be possible, by an extension of the theory, to investigate the variation in *intensity* of an odour with its concentration in the air about the threshold value. The intensity of an odour, however, is difficult to define or to measure for humans, and little work has been done on this subject. Experiments on *Phormia* or other insects seem more suitable for work on this parameter. The recent electrophysiological experiments of Beidler (1958) have shown that the activity of the olfactory receptors in the rabbit increases with the concentration of odorant until a maximum level of activity is reached.

It is not clear, however, whether excitation of an olfactory cell by an odorant is an all-or-nothing phenomenon (so that the intensity of smell depends on the number of cells stimulated), or whether there are different degrees of stimulation

of one cell (when the intensity would depend on the number of impulses per second, *i.e.*, on the number of small areas per cell occupied by p odorant molecules).

Repeated exposure of the receptors to concentrations of odorant well above the threshold causes a temporary loss of sensitivity. On the theory of olfaction proposed here, this represents a simple depolarization phenomenon, the cell being unable to build up the "equilibrium" ion concentrations in the periods between successive inspirations. This phenomenon of fatigue or adaptation has been discussed by Adrian (1950a) with reference to olfaction in the rabbit and in general (1950b). Kristensen and Zilstorff-Pedersen (1953) discuss olfactory fatigue in man.

Two problems concerning the qualitative aspect of odour remain. Is there a relation between the adsorption and morphology of an odorant molecule and the type of odour it possesses? Secondly, is there more than one type of receptor in the olfactory epithelium?

The first point has been considered recently by Amoore (1952) and Timmermans (1954); the general conclusion is that molecular shape is important in determining the qualitative characteristics of an odorant. Amoore correlated the type of an odour with the shapes and electronic configurations of the molecules whilst Timmermans suggested that all molecules smelling like camphor are spherical, and conversely, that all spherical molecules have a camphorous smell. Unfortunately, there are exceptions to this rule which would otherwise be of the greatest significance.

In an exhaustive survey of work on the morphology of the olfactory region in vertebrates, Allison (1953) concludes that attempts (of this nature) to discover two or more types of olfactory receptor have been unsuccessful. Le Gros Clark (1957), however, has noted histological differences between receptor cells in different regions of the olfactory epithelium and has considered the possibility that this is linked with the problem of differential sensitivity.

Adrian (1950c) noted from his studies of the electrical activity of the olfactory bulb during stimulation that, since the receptors of lower animals are located in folds and are not equally accessible to currents of air, discharges set up by different smells may differ greatly in their temporal and spatial pattern. This fact alone is hardly sufficient to account for olfactory specificity since the olfactory epithelium in some higher animals, including man, is comparatively flat and exposed over its entire surface. His more recent electrophysiological investigations (1951, 1952, 1954) indicated that there are several types of olfactory receptor but that they are specifically sensitive at concentrations only just above the threshold; at concentrations much above this they react to more odorants. Beidler and Tucker (1955) recorded neutral activity from isolated bundles of primary olfactory nerves of the opossum. Their results, too, yielded evidence for a certain degree of specificity in the receptors. These techniques, however, because of the difficulties of placing the electrodes, yield indirect and imprecise information about different receptor types. We may conclude (with Allison) that any observed differences in receptor response must be attributed to subtle differences in structure at the physico-chemical level. Certainly, the specificities of the receptors might depend on the ease with which the different membrane layers are distorted: slight differences in their structure could render them more readily disoriented by molecules of certain shapes than by others. If there are only a few types of

olfactory receptor, however, then finer shades of odour must be the result of a complex pattern of impulses arriving in the brain as a result of the different intensities of stimulation of different receptor types as suggested by Adrian. Mullins (1955) and Cheeseman and Mayne (1953) have studied this question with regard to olfaction in humans, by examining the interference of one odorant with the threshold of another. Mullins suggests that since butanol does not disturb the threshold for butane and vice versa, there are at least two types of receptor membrane in the olfactory epithelium.

The authors wish to express their gratitude to Sir Eric Rideal, F.R.S., for helpful advice during the course of this investigation.

Summary

1. It is proposed that a stimulus is initiated in an olfactory receptor cell only when a critical number (p) of odorant molecules is concentrated within one small area of the cell membrane. An equation is derived which relates the olfactory threshold for humans to this number p and to the adsorption constant for molecules passing from air to the oil/water interface. Olfactory thresholds are calculated for a range of odorants on the assumption that $1/p$ is a function of the molecular cross-section area of an odorant. The calculated thresholds agree with the observed values; that predicted for glycerol exceeds the saturation concentration in the air so that this substance is odourless.

2. The equation suggests that olfactory thresholds should increase as the temperature is raised, as has been found experimentally. The results suggest that the olfactory cell membrane is lipoid in nature; the calculated "active surface area" of each olfactory cell is less than the observed total value. The effectiveness of compounds as odorants for *Phormia* and dogs, as well as for humans, depends on the concentration adsorbed on the membrane and upon the shape and size of the odorant molecules. Contact chemoreception in *Phormia*, however, is dependent only upon the appropriate adsorption constant and not upon molecular morphology.

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THE PHYSIOLOGY OF SKELETON FORMATION IN CORALS. II. CALCIUM DEPOSITION BY HERMATYPIC CORALS UNDER VARIOUS CONDITIONS IN THE REEF

THOMAS F. GOREAU [1] AND NORA I. GOREAU

Department of Physiology, University College of the West Indies and The New York Zoological Society

In a previous paper (Goreau, 1959a) a method was described for the direct determination of calcium deposition in madreporarian corals and other calcareous reef building organisms by the use of radioactive calcium-45. In these initial experiments calcification rates were measured under a variety of controlled conditions in the laboratory, but no information was obtained as to whether these were comparable with growth rates of corals in the open reef. Preliminary results suggested that growth in the natural environment is faster than that which we determined under laboratory conditions. In order to study this possibility more extensively we modified our method to allow direct assessment of calcium deposition in hermatypic corals under conditions approximating those found in the reef. For test organisms we selected fifteen of the most important West Indian shallow water hermatypic coelenterates, among which were included thirteen Madreporaria and two of the Milleporina.[2] These species were all collected from actively growing shallow reefs near Lime and Rackham Cays, located respectively two and four miles southeast of Port Royal, Jamaica, W. I. A general review of the abundance and distribution of these corals together with a description of Jamaican coral reefs has been published elsewhere (Goreau, 1959b).

Procedure

All the experiments were conducted in the reefs mentioned above and the necessary equipment was brought to the site in a power launch which was moored in a suitable shallow place in about five or six feet for the duration of the operation. A boatman and two divers were required to carry out the various steps. Large twelve-liter Pyrex vacuum desiccator jars containing plastic-wrapped lead weights were used as the experimental vessels. These were sunk on the reef and selected coral colonies were gently detached by one of the divers who then transferred them into the jars in such a way that the living surface of the coenosarc was not

[1] Mailing address: Department of Physiology, University College of the West Indies, Mona, St. Andrew, Jamaica, B. W. I.

[2] These species were *Acropora palmata* (Lamarck), *A. cervicornis* (Lamarck), *Porites porites* (Pallas), *P. furcata* (Lamarck), *P. astreoides* (Lamarck), *Siderastrea siderea* (Ellis and Solander), *S. radians* (Pallas), *Diploria clivosa* (Ellis and Solander), *D. strigosa* (Dana), *D. labyrinthiformis* (Linnaeus), *Colpophyllia natans* (Muller), *Manicina areolata* (Linnaeus), *Montastrea annularis* (Ellis and Solander), *Millepora alcicornis* (Linnaeus), *M. complanata* (Lamarck). The classification of the Madreporaria is according to Wells (1956), that of the Milleporina is according to Boschma (1948).

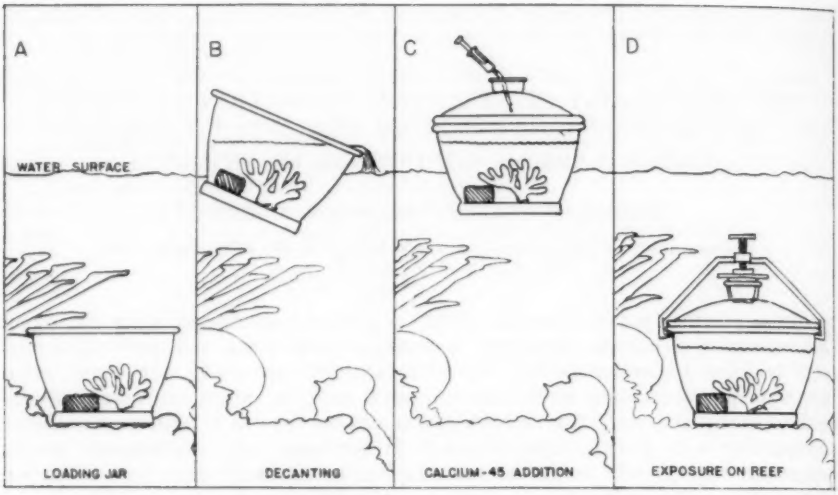

FIGURE 1. Procedure for loading and setting out the experimental jars on the reef. The cross-hatched square object on the bottom of the vessel is a plastic-wrapped lead brick used to weigh down the jar and stabilize it against currents and wave surge.

touched. The entire procedure was carried out under water and the coral colonies did not come in contact with air at any stage of the experiment. For replicate runs on the same species, colonies of similar size and shape were used. After loading, the jars were briefly raised above the surface, the excess water decanted, and ten milliliters of sea water containing approximately 0.2 mc. Ca^{45} Cl_2 adjusted to pH 8 were added. The lids were then put on, the jars gently swirled to mix the contents and a water sample taken for counting. The joint was sealed with silicone stopcock grease and the lids were secured by a bow clamp. The entire loading procedure is shown in Figure 1. Some of the jars were painted black to exclude light and one of these was run in each series. The full jars were placed by the divers on the reef at or near the sites from which the corals within had originally been taken, and situated so that there was no shading by neighbouring coral colonies. The standard depth was five feet, but several runs were also made at depths ranging from one to twelve feet. During our experiments the water temperatures were between 26° C. and 28° C. with fluctuations of not more than one degree centigrade during individual runs. Relative light intensities were estimated with a waterproof light meter. Although this instrument was not suitable for measurement of incident light, it could be used to give comparative light values by pointing it horizontally at a matte white surface about one foot distant.

The experiments were allowed to run for four to eight hours through the middle of the day from about 9:00 A.M. onward. They were terminated by picking up the jars from the reef, taking a water aliquot for counting, and transferring the corals to large volumes of fresh non-radioactive sea water with a pair of long tongs. The time of removal from the radioactive sea water was taken as the end point of the

experiment. The dark jars were allowed to run for only about four hours, and opened first to minimize pH changes due to anaerobiosis.

The living colonies were rinsed for two hours in running fresh sea water to remove adhering radioactivity from the coenosarc. Ramose corals were sampled by the method described in our previous paper (Goreau, 1959a). Massive non-branching colonies were first split into smaller pieces with a cold chisel, cutting the samples from the surface in such a way as to include the whole thickness of the polypary layer.

A hollow steel core punch was used to obtain samples having a uniform cross-sectional area of 2 cm^2. With a hammer the punch was driven in several centimeters so that the entire thickness of the coenosarc was included. Care was taken to exclude from the sample any boring sponges, worms, clams, Crustacea and encrusting organisms. A shield and gloves were used to prevent contamination by radioactive coral fragments. Different parts of colonies were sampled to measure any growth gradients. The resultant cores were extruded by a piston, the method being illustrated in Figure 2. Cores from the deeper layers, just beneath the coenosarc, were taken to measure the diffusion of Ca-45 into the non-living parts of the skeleton. The coring method was not used on such corals as *Acropora cervicornis* and *Porites furcata* because of the relatively small size of their branches. These were therefore sampled by cutting or hack-sawing off pieces with accurately known dimensions from which the approximate surface areas could be calculated with suitable formulae. Measurements and sketches were made of all colonies treated in this way to locate the areas from which the samples had been taken. As these pieces were often large, some of them reaching several grams in weight, they were individually dissolved in numbered conical flasks containing 20 ml. dilute HCl (1:1).

After solution, all samples were heated, homogenized with a Potter tissue

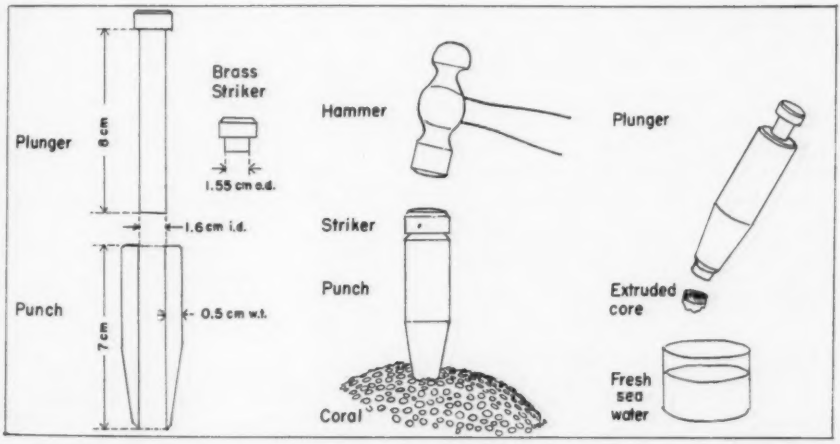

FIGURE 2. The core punch and the procedure for taking coral samples of known cross-sectional area.

TABLE I

Calcium deposition by hermatypic corals

Family and species	Branching or massive	Calcium uptake in μg. Ca/mg. N/hr. Sample numbers in parentheses			Maximum light:dark ratio
		Sunshine	Cloudy	Darkness	
Acroporidae					
A. palmata	B*	43.6 ± 9.81 (8)	26.3 ± 7.5 (10)	3.2 ± 0.64 (7)	13.5
		50.9 ± 8.40 (9)		4.0 ± 0.51 (8)	12.8
*A. cervicornis**	B*	53.7 ± 7.43 (9)	39.4 ± 8.65 (7)	8.4 ± 0.66 (6)	6.4
		71.5 ± 12.92 (5)	33.2 ± 6.80 (6)	4.1 ± 0.35 (8)	8.5
		61.0 ± 8.78 (6)			
		73.7 ± 18.82 (6)			
Poritidae					
*P. porites**	B*	25.1 ± 3.90 (9)		7.9 ± 4.60 (9)	3.2
		18.6 ± 3.31 (13)			
		23.2 ± 3.54 (8)			
		20.7 ± 1.73 (8)			
		29.3 ± 4.91 (8)			
		27.8 ± 5.58 (15)			
P. furcata	B*	15.4 ± 3.70 (8)		1.9 ± 0.20 (6)	8.1
		17.8 ± 4.25 (9)			
P. astreoides	M	6.7 ± 3.65 (8)		1.5 ± 0.36 (5)	4.5
		6.8 ± 1.35 (5)			
		13.7 ± 4.36 (8)			
		10.1 ± 2.20 (7)			
		5.5 ± 1.55 (7)			
		14.1 ± 3.00 (9)			
Siderastreidae					
S. siderea	M	13.7 ± 3.02 (11)			
		9.2 ± 2.50 (12)			
S. radians	M	7.1 ± 1.34 (6)			
Faviidae					
D. clivosa	M	6.7 ± 1.27 (8)			
		6.5 ± 1.00 (8)			
D. strigosa	M	6.5 ± 1.38 (11)			
		5.2 ± 1.11 (12)			
D. labyrinthiformis	M	20.9 ± 5.80 (15)			
		14.8 ± 2.72 (14)			
C. natans	M	14.9 ± 3.80 (11)			
		10.4 ± 1.85 (8)		2.5 ± 1.56 (8)	4.3
M. areolata	M				
zooxanthellae		13.4 ± 7.54 (10)		1.8 ± 0.42 (9)	7.5
no zooxanthellae		0.7 ± 0.31 (15)			

TABLE I—*Continued*

Family and species	Branching or massive	Calcium uptake in μg. Ca/mg. N/hr. Sample numbers in parentheses			Maximum light:dark ratio
		Sunshine	Cloudy	Darkness	
M. annularis	M	7.3 ± 1.71 (10) 8.9 ± 2.00 (10) 11.7 ± 2.00 (12)		0.3 ± 0.05 (9)	22.9
Milleporidae					
M. complanata	B*	35.7 ± 3.89 (9) 47.1 ± 13.80 (12) 36.2 ± 4.76 (6) 36.5 ± 3.92 (11)	24.6 ± 5.52 (9) 23.2 ± 4.43 (10)	4.7 ± 0.36 (10)	7.5
M. alcicornis	B*		23.6 ± 3.52 (8)		

* Apical polyps only.

grinder, cooled, and diluted to twenty-five milliliters with distilled water. Replicate aliquots were taken for counting and nitrogen analysis. The radioactivity was determined by the method described in our previous paper and the total amount of calcium taken up was calculated from the specific activity of calcium-45 in the sea water samples. The final calcification rate was expressed either in μg. Ca deposited per hour per mg. N, or μg. Ca deposited per cm^2. per hour. The final results were not corrected for isotopic exchange since the calcium-45 incorporated in this way amounted to a maximum of 0.5 per cent of the total calcium deposited.

RESULTS AND OBSERVATIONS

The highest calcification rates were invariably found in the terminal regions of the branching, or ramose corals, whereas lower rates were characteristic for the massive non-branching corals. Our results, in terms of calcium deposited per hour per milligram of organic nitrogen, are brought together in Table I. In decreasing order, the species we tested ranked approximately as follows: *A. cervicornis, A. palmata, M. complanata, M. alcicornis, P. porites, P. furcata, D. labyrinthiformis, C. natans, M. areolata, P. astreoides, A. siderea, M. annularis, S. radians, D. clivosa, D. strigosa.* The first six species are branching, the others are all massive non-branching.

As the ambient light intensity was previously shown to have an effect on the calcification rates of reef corals (*cf.* Goreau, 1959a), we tested the influence of different light conditions on coral calcium uptake in the reef by running some of our experiments on cloudy days, and some in complete darkness. Because exact measurements of the underwater light intensities could not be made due to lack of a suitable meter, we have listed our results in Table I under arbitrary headings, depending on whether the light conditions during the experiments were clear and sunny, cloudy or no light at all. In all species tested, the calcification rate was highest during sunny weather, lower during cloudy weather, and very low in darkness. The maximum light: dark ratios varied over a broad range, between approximately 3.2 to 22.9.

Previously we have also presented evidence that the calcification rate of corals is in part dependent on the presence of zooxanthellae, showing a marked fall in their absence (Goreau, 1959a). An opportunity came to repeat this experiment under natural conditions when several bleached and zooxanthella-less colonies of *M. areolata* were found alive and in good condition, growing unattached in semi-darkness under a large hollow coral head. The polyps of these were expanded, and their tissues were colourless and translucent. Controls were run at the same time with normal yellowish-brown colonies collected nearby. The results, which are included in Table I, show that the normal coral with zooxanthellae calcified about nineteen times faster than those which had lost their algae. This is in substantial agreement with our previous experiments. The light : dark ratio for normal colonies of *M. areolata* was about 7.5, which means that colonies with zooxanthellae could still calcify considerably faster in darkness than could zooxanthella-less colonies in the light. An hypothesis concerning a mechanism whereby the calcification process

TABLE II

Calcification rates in different parts of branches of A. cervicornis at various light intensities

Light conditions on the reef	Calcium uptake in μg. Ca/mg. N/hr. Sample numbers in parentheses		
	Apical polyps	2 cm. behind apex	3 cm. behind apex
Darkness	8.4 ± 0.66 (6)	2.2 ± 0.72 (6)	
Cloudy weather	39.4 ± 8.65 (7)	15.1 ± 6.20 (8)*	
	33.2 ± 6.80 (6)	9.8 ± 3.91 (5)*	
Bright sunshine	53.3 ± 7.43 (9)	33.9 ± 3.47 (10)	27.5 ± 4.45 (7)
	71.5 ± 12.92 (5)	48.8 ± 8.39 (4)	28.9 ± 1.00 (5)
	61.0 ± 8.78 (6)	42.5 ± 7.15 (7)	31.6 ± 5.39 (7)
	73.7 ± 18.82 (6)	36.8 ± 6.57 (5)	25.7 ± 3.61 (6)

* Samples taken 3 cm. behind apical polyp.

in Madreporaria may be stimulated by photosynthesizing zooxanthellae has been published in the paper cited above.

Considerable variance in the calcification rates is evident in our results, both within sample groups taken from the same colony, as shown by the standard deviation of the means, and between different colonies of the same species. To determine the probability that these two forms of variance were due to dissimilar causes, the data from four species [3] were analysed by the F test (*cf.* Snedecor, 1946; pp. 232–233). This shows that the F values varied from less than one to less than five per cent, thus indicating that differences in calcium uptake rate between uniformly sized colonies of the same species growing under similar conditions were significant. Similar variances almost certainly occur in most of the other species tested.

Preliminary tests to determine the effect of depth were carried out on three coral species simultaneously at 1, 3, 6, 9, and 12 feet. The results were inconclusive, showing no correlation of the calcification rate with depth over the range

[3] *A. cervicornis, P. porites, P. astreoides, M. complanata.*

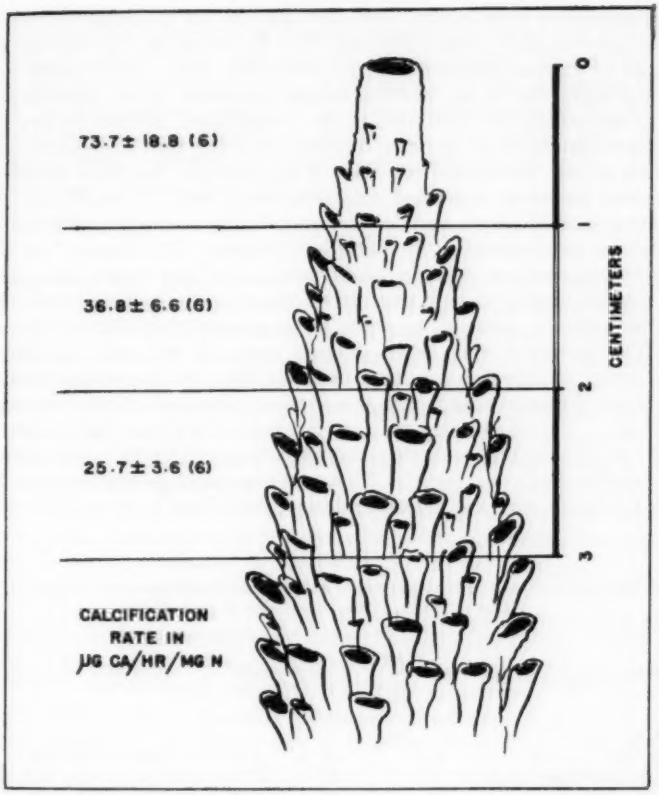

FIGURE 3. Apical region of a branch of *Acropora cervicornis* showing the existence of a calcification gradient. The large terminal polyp at the top has the highest rate of calcium incorporation.

tested probably because the light intensity was more or less uniform due to back-scatter by sandy patches in that part of the reef where these experiments were made. Further work correlated with more precise measurements of the incident light intensity over a greater depth range is now in progress.

Calcification rates in the ramose corals tended to decrease systematically from a maximum in the apical polyps to much lower rates in the lateral and basal branch corallites. A characteristic gradient is shown in Table II and Figure 3 for the staghorn coral, *A. cervicornis,* where the calcification was measured at one-centimeter intervals away from the branch apex. Systematic variations in the calcification rates were also observed in other branching species, *e.g., A. palmata, M. complanata* and *P. furcata* as shown in Table III. In the massive corals tested, gradients were absent and such variations as were observed appeared to be random over the entire colony.

Some preliminary experiments were also carried out to determine the calcification rate in terms of surface area. This was done by measuring the calcium uptake in core samples of known diameter and cross-sectional area. Our results are almost certainly too high, due to an uncompensated systematic error introduced by the difficulty of measuring the total area of the complicated skeletal surface of corals. Pending development of an accurate method for doing this, a problem now under investigation in our laboratory, the area of our samples has been calculated from their geometry based on smoothed out dimensions, and our results can therefore only be interpreted in very approximate terms on the assumption that all errors in the method are predominantly in the same direction. In column 1 of Table III are shown the approximate calcium uptakes per cm.^{2} in four ramose and two massive corals. It is interesting to note that the calcification rate per unit area of the two non-branching corals seems to be equal to or greater than that of the branching species. The reverse is true when calcium uptake is estimated in terms of the nitrogen content as shown in column 3 of Table III. In the second column of this table are listed the approximate tissue biomasses contained in the sample in terms of mg. N/cm^2. This shows that the two massive faviid corals had a higher content of organic matter per unit area than the branching acroporid, poritid, and hydrocoralline species that were tested. This confirms what is readily seen by naked eye, that the acroporid and hydrocoralline corals have a very thin translucent

TABLE III

The relationship of approximate geometric area to protein nitrogen content and calcium uptake in corals. Sample number in parentheses

Species and location of samples	Branching or massive form	Approximate μg. Ca/hr./cm.^{2}	Approximate mg. N/cm.^{2}	μg. Ca/mg. N/hr.
A. palmata	B			
Outer edge of frond		69 ± 9.5 (3)	2.4 ± 0.4 (3)	29.5 ± 0.78 (3)
7 cm. behind edge, upper		52 ± 2.0 (3)	3.0 ± 0.3 (3)	17.8 ± 2.59 (3)
7 cm. behind edge, under		32 ± 4.0 (3)	1.8 ± 0.7 (3)	17.5 ± 0.05 (3)
A. cervicornis	B			
Apical polyps		20 ± 1.7 (9)	0.5 ± 0.05 (9)	41.7 ±11.7 (9)
3 cm. behind apex		12 ± 1.8 (6)	0.6 ± 0.01 (6)	17.4 ± 2.95 (6)
5–7 cm. behind apex		10 ± 1.6 (3)	0.8 ± 0.4 (3)	13.8 ± 0.84 (3)
M. complanata	B			
Superior edge of frond		38 ± 5.7 (9)	1.1 ± 0.1 (9)	35.7 ± 3.89 (9)
10 cm. behind edge		12 ± 1.8 (4)	2.0 ± 0.3 (4)	5.6 ± 0.22 (4)
P. furcata	B			
Apical 1 cm. of branch		36 ± 6.7 (8)	2.4 ± 0.1 (8)	15.4 ± 3.70 (8)
2 cm. behind apex		6 ± 1.4 (3)	3.3 ± 0.9 (3)	3.34 ± 0.94 (3)
5 cm. behind apex		4 ± 2.9 (2)	3.1 ± 0.6 (2)	3.07 ± 0.55 (2)
C. natans	M			
Random over whole colony		80 ± 1.2 (8)	7.9 ± 1.2 (8)	10.4 ± 1.85 (8)
M. annularis	M	54 ± 11.9 (3)	7.3 ± 0.6 (5)	7.32 ± 1.71 (5)

coenosarc; the poritids a somewhat thicker, more opaque one; and that the faviids on the whole are rather fleshy, especially the species analysed here. The possibility thus arises that the calcification powers of the massive corals, which are less marked than those of the branching species on the basis of tissue mass as expressed by the nitrogen content, may be as great or greater per unit surface area of the basal calicoblastic epidermis which is the organ of skeletogenesis in corals. The problem is now under more detailed investigation.

Discussion

The long-term field observations of Wood-Jones (1910), Mayor (1924), Edmondson (1929) and the Stephensons (1933) directed attention to the fact that corals do not necessarily grow at even rates. The experiments of the Stephensons were particularly valuable as they established that individual variations among healthy and normal corals of the same species growing for the same length of time in the same environment gave very irregular results, leading to the conclusion that the use of averages was preferable to individual figures. Our data seem to corroborate this view to the extent of showing that there are significant variations in calcification rates among similar colonies of the same species under identical environmental conditions over the comparatively short periods of time during which our measurements were made. It is, however, uncertain whether the variances we observed in experiments lasting only a few hours are similar to those described in the studies of other investigators, all of which were made under non-uniform conditions over periods of months to years. It should be understood that because of the dissimilarity in aims, methods and expression of results, the calcification rates cited in this paper cannot be compared with the coral growth data obtained by previous observers who used completely different procedures. We have endeavoured to determine calcium uptake of corals and to use this as an index of physiological function, not as a direct measure of absolute growth rate. We hope nevertheless that the hiatus between these two objectives will disappear with further work.

The present results also support our earlier observations (Goreau, 1959a) that the calcification rates of individual corallites fluctuate widely, and probably at random, even in symmetrical colonies. Statistically, this type of variance appears to be separate and distinct from the clonal growth differences that occur among individual colonies in a population of a given coral species.

The local fluctuations in the calcium uptake of individual corallites may be related to the manner in which the coral skeleton is laid down and organised. The sclerenchyme, or skeleton, consists of a succession of discontinuous layers held together by walls and partitions: the dissepiments, thecae and sclerosepta, respectively. The dissepiments are separated from each other by more or less regular horizontal spacings which suggests that the process of deposition is itself discontinuous, possibly involving local and temporary detachment of the calicoblast from the skeletal basis just before the secretion of a new layer, a millimeter or so above the old. Circumstantial evidence that the attachment of the polyp to its skeleton may be under some degree of facultative control is provided by the observation that polyps of starving corals are able to detach themselves completely from the corallum. In this phase they can stay alive for some weeks without showing any

evidence of renewed skeletogenesis although they are able to ingest food normally (Goreau, unpublished observations). We do not suggest that the coenosarc ever becomes entirely detached under normal circumstances, but the fact that it is able to do so at all indicates that the polyps are not anchored down quite as securely as is at present believed. On the basis of mineralogical evidence, Bryan and Hill (1941) have also suggested that madreporarian calcification is a discontinuous process involving possible temporary detachment of parts of the polyp.

TABLE IV

Comparison of calcification rates of some hermatypic corals with their relative abundance on a large Jamaican coral reef

Family and species	Relative abundance in all zones of reef	Relative calcification rate
Acroporidae		
A. cervicornis	+++	oooooo
A. palmata	++++	ooooo
Poritidae		
P. porites	++	oooo
P. furcata	+++	oo
P. astreoides	++++	o
Siderastreidae		
S. siderea	++	oo
*S. radians**	++	o
Faviidae		
D. clivosa	++	o
D. strigosa	+++	o
D. labyrinthiformis	+	ooo
C. natans	++	oo
*M. areolata**	+	oo
M. annularis	++++	oo
Milleporidae		
M. complanata	+++	ooooo
M. alcicornis	+ (?)	ooo

Legend:			
+	not common	o	0– 9 μg. Ca/mg. N/hr.
++	common	oo	10–19 μg. Ca/mg. N/hr.
+++	abundant	ooo	20–29 μg. Ca/mg. N/hr.
++++	very abundant and dominant in some zones	oooo	30–39 μg. Ca/mg. N/hr.
		ooooo	40–49 μg. Ca/mg. N/hr.
		oooooo	50+ μg. Ca/mg. N/hr.

* Colonies numerous but of small size with low aggregate biomass.

We have so far found no consistent relationship between calcification rates of corals in terms of organic matter, and their prevalence on a coral reef. In Table IV the relative calcification rates of the species tested are compared with the relative abundance of the same corals on a large Jamaican reef. For the Acroporidae and Milleporidae, which are the predominant hermatypes of the shallowest reef zones, there may be some correlation of abundance with calcium uptake. In the Siderastreidae, the much larger and more common *S. siderea* appears to calcify faster than

the smaller but hardier *S. radians*. However, in the Poritidae, *P. astreoides* is the more frequent and important species in Jamaican reefs, although it calcifies much less rapidly than its branching congeners *P. porites* and *P. furcata*. In the Faviidae, the apparent discrepancy between the occurrence on the reef and calcification rate is still more pronounced since the faster calcifying species such as *C. natans, D. labyrinthiformis* and *M. areolata* are less prevalent on the reef than the slower growing *D. strigosa, D. clivosa* and *M. annularis*.

Although the preeminence of the massive corals may in part be due to the greater susceptibility of branching corals to damage by heavy seas, this fails to explain why branching corals are in general typical of the turbulent shallow waters whereas the massive corals are more characteristic of the deeper calmer regions of the reef. Despite the fact that the growth rate of the massive coral *M. annularis* (*cf.* also Vaughan, 1915, and Vaughan and Wells, 1943, p. 64) is relatively low, in Jamaica this species is nevertheless one of the most important hermatypes in regard to the total quantity of calcareous material contributed to the framework of the reef. It is usually the dominant coral in the deeper regions of the fore-reef, reaching a peak of development in the buttress zone where the gigantic size of its colonies is evidence of extremely vigorous proliferation (Goreau, 1958a, 1959b). This zone is named for the great coral promontories which grow upward and forward into deeper water, forming immense dentate projections that are oriented with their long axis into the prevailing seas so that they present a maximal area for coral growth with a minimal frontal area exposed to the surge of the waves. In the buttress zone the growth habit of such massive corals as *M. annularis* and *P. astreoides* becomes curiously flattened so that the colonies often resemble platters, roof shingles or great flow sheets, whereas in other shallower zones in which these corals are frequently much more exposed to surf and currents their colonial habit is mushroom shaped. If it is assumed on the basis of our preliminary data that the growing power of reef corals is proportional to the area of the calicoblast, then the formation of buttresses and a flattened growth habit may be interpreted as representing adaptive modifications which enable massive corals to compete more successfully on an area basis with the faster growing ramose corals.

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Summary and Conclusions

1. The calcium uptakes of 13 hermatypic corals and 2 hydrocorallines were measured by a modified calcium-45 method under conditions approximating those of the natural environment of the reef in experiments lasting four to eight hours.

2. When measured on the basis of nitrogen content, the growth rates of the branching corals were higher than those of the massive corals. On the basis of area, however, the latter appear to grow as fast or faster than the former.

3. Light intensity had a profound influence on the growth rate under the conditions of our experiments. All corals tested deposited calcium fastest in sunlight, less during cloudy weather and least in darkness. Bleached zooxanthella-less colonies deposited calcium at lower rates in the light than normal colonies with zooxanthellae did in darkness.

4. Systematic calcification gradients were observed in branching corals but not in massive species.

5. Although there was a considerable variance in the growth rate from place to place within individual colonies, we also observed large and significant differences in the growth rate between individual colonies of the same species, size and shape under similar conditions.

6. It is suggested that the organisation of the skeleton, which is really composed of many separate lamellae with spaces in between, indicates that the calcification process itself may be discontinuous, and that this may in turn be responsible for the growth fluctuations that were observed within the coral polyp populations of individual colonies.

7. No hard and fast correlation was observed between the calcification rates per unit of nitrogen and the relative abundance of the species on the reef. Although some of the commonest shallow water corals are very fast calcifiers as well, the most important West Indian reef builders are the slower growing massive corals.

8. An explanation is put forward to the effect that growth of massive corals in the reef is enhanced by the formation of buttresses which serve to increase the available surface area for calcification.

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DISTRIBUTION OF A RADIOMERCURY-LABELLED DIURETIC (CHLORMERODRIN) IN TISSUES OF MARINE FISH [1]

ROGER L. GREIF AND MARILYN DU VIGNEAUD

Department of Physiology, Cornell University Medical College, New York City, and the Marine Biological Laboratory, Woods Hole, Mass.

The ability of mercurial compounds to increase urine flow, recognized at least since the time of Paracelsus (1493–1541), has been widely applied in clinical medicine following the introduction of the organic mercurial diuretics (Saxl and Heilig, 1920). The association of suppression of urine flow with poisoning due to inorganic mercury compounds (Woodman and Tidy, 1877) also suggests that renal tissue is particularly susceptible to the action of such substances. Ludwig and Zillner (1890) were among the early investigators who demonstrated by chemical analysis that the kidneys of dogs poisoned with mercuric chloride contain far higher concentrations of mercury than other tissues. Many subsequent studies, with the use of both biochemical and radioisotopic methods, have confirmed this finding.

Hg^{203}-labelled chlormerodrin has recently been used to determine more precisely the location of mercury deposition in mammalian kidney. In dogs and in rats the concentration is highest in the outer renal cortex, decreases in the medulla, and is lowest in renal papillary tissue (Greif *et al.*, 1956). In this connection, Walker and Oliver (1941) have pointed out that the rat outer renal cortex is composed largely of proximal segment of the tubule. The purpose of the present study was to determine, with the use of a radiomercury-labeled organic mercurial diuretic, whether mercury is selectively concentrated in the kidneys of marine fish with a variety of nephron types.

Materials and Methods

Specimens of flounder (*Paralycthys dentatus*), toadfish (*Opsanus tau*) and dogfish (*Mustelus canis*) were furnished by the Collecting Department of the Marine Biological Laboratory, and were stored briefly in live cars before use. They were weighed and injected with alkaline solutions of chlormerodrin [2] in concentrations indicated in the tables. Most injections were made intramuscularly in the fleshy part of the tail, although a few intramuscular injections were placed immediately dorsal and posterior to the gills. The fish were placed in a running sea water aquarium until sacrificed. At the time of killing, the fish were stunned and bled from either the tail vein or the heart with a needle attached to a heparinized syringe. Tissue was then rapidly removed from the fish, weighed on a torsion balance, and transferred to a test tube for counting in a well-type scintillation counter with scaler. Blocks of tissue were removed from the anterior, medial, and

[1] This investigation was supported by Grant A-786 of the National Institute of Arthritis and Metabolic Diseases, USPHS, Bethesda, Md.

[2] Generously provided by Dr. R. F. Pitts.

posterior portions of the kidney for counting. Results are expressed as milligrams chlormerodrin mercury per gram wet weight of tissue. Unless otherwise indicated, the middle portion of the kidney was selected for comparison with other excised tissues. Urine was obtained either by inserting a pipette into the urogenital papilla or aspirating the exposed bladder with a syringe and needle. Phenol red accumulation in the excised flounder tubule was studied by the method of Forster and Taggart (1950).

Results

a) *Flounder.* Under the present experimental conditions a high tissue mercury concentration is always found in the kidneys. This is especially striking at the dosage level of one milligram chlormerodrin mercury per kilogram body weight (Table I), an amount which will produce diuresis in mammals (Borghgraef *et al.*, 1956). The larger dose, 10 milligrams mercury per kilogram (Table II), which is toxic to mammals, also proved fatal to some of the fish. Kidney tissue excised from flounder surviving the larger dose was unusually friable. On microscopic examination, the freshly teased tubules appeared cloudy and granular and failed to concentrate phenol red. When such tissue is fixed in Susa fluid and stained with hematoxylin and eosin it cannot be readily distinguished from kidney of fish given

Table I

Mean tissue concentration of radiomercury administered as one milligram labelled chlormerodrin mercury per kilo body weight. All values expressed as micrograms mercury/gram wet weight of tissue

Species	No. of Animals	Plasma	Kidney	Liver	Bile	Gills	Heart	Stomach	Gonads	Spleen
12 Hours After Intramuscular Injection										
Flounder	2	0.7	52.2	3.2	9.7	0.2	0.5	0.4	0.2	0.7
Toadfish	1	0.8	13.2	0.3	0.0	0.5	0.0	0.5	0.0	0.2
Dogfish	1	0.9	42.2	4.4	21.9	0.0	0.0	0.3	0.8	0.5
24 Hours After Intramuscular Injection										
Flounder	3	0.3	80.1	3.9	1.7	0.2	0.4	0.2	0.2	0.5
Toadfish	2	0.7	12.0	0.3	0.2	0.5	0.5	0.4	0.1	1.0
Dogfish	3	0.6	14.5	4.0	33.6	0.4	0.2	2.0	0.9	0.4
48 Hours After Intramuscular Injection										
Flounder	2	3.5	35.6	6.7	2.8	1.9	0.2	0.4	0.3	1.0
Toadfish	2	0.3	9.0	0.4	0.2	0.3	0.3	0.2	1.2	0.4
Dogfish	1	0.2	6.4	2.7	21.0	0.9	0.3	2.9	0.7	0.4
72 Hours After Intramuscular Injection										
Flounder	2	0.1	24.6	5.7	2.7	0.2	0.2	0.0	0.2	0.7
Dogfish	1	0.1	4.4	7.3	114.0	1.0	0.2	0.4	0.7	0.4

TABLE II

Mean tissue concentration of radiomercury after administration of 10 milligrams labelled chlormerodrin mercury per kilo body weight. All values expressed as micrograms mercury/gram wet weight of tissue

Species	No. of Animals	Plasma	Kidney	Liver	Bile	Gills	Heart	Stomach	Gonads	Spleen
12 Hours After Intramuscular Injection										
Flounder	4	13.6	288.0	55.6	12.8	12.5	8.2	8.7	7.3	30.2
Toadfish	2	29.5	197.0	15.0	2.6	12.3	23.5	11.7	6.4	16.5
24 Hours After Intramuscular Injection										
Flounder	4	11.9	146.5	51.8	53.7	11.4	9.3	6.0	6.4	30.1
Toadfish	3	19.7	128.9	29.2	20.0	10.4	21.2	12.0	8.4	13.6
48 Hours After Intramuscular Injection										
Flounder	3	12.6	77.1	59.2	121.0	10.3	8.6	8.5	9.8	37.5
Toadfish	3	19.0	105.0	36.2	37.8	11.0	21.4	10.2	5.0	16.6
72 Hours After Intramuscular Injection										
Flounder	2	10.2	89.4	45.1	57.0	7.1	4.2	7.7	6.7	19.6
Toadfish	3	20.8	106.5	40.9	55.7	11.7	29.2	11.5	18.5	15.8

the smaller dose of mercurial or from that of control flounder, although these latter tissues concentrated phenol red and appeared normal when viewed with the dissecting microscope. On the regimen of 10 milligrams Hg per kilogram, high concentrations of mercury are seen not only in the kidney but also in the liver and bile of the surviving animals.

b) *Toadfish.* In Table I and Table II may be found the distribution of chlormerodrin mercury in the tissues of this species. As in the flounder the mercury concentration in the kidney is high, but at 10 milligrams mercury per kilogram the concentration in the bile in toadfish does not exceed the concentration in the kidney. It was possible to obtain blood and urine samples in 11 toadfish after chlormerodrin injection. Despite great individual variation, in all but one animal the urine-to-plasma ratio was at least 4, and the mean value for this ratio was 30.7 ± 9.6 (S. E.). Phenol red, injected with the chlormerodrin, also appeared in the urine.

c) *Dogfish.* Tissue mercury distribution in a small number of animals is included in Table I. The most striking difference to be seen in this instance is the high mercury concentration in the bile on the one milligram mercury per kilogram body weight regimen.

General. In all animals at the one milligram/kilogram dose the kidney mercury concentration is high, and only in the dogfish is the concentration in the liver and in the bile impressive. It is notable that in none of the animals on any regimen is the mercury concentration in either the gills or the stomach higher than in the

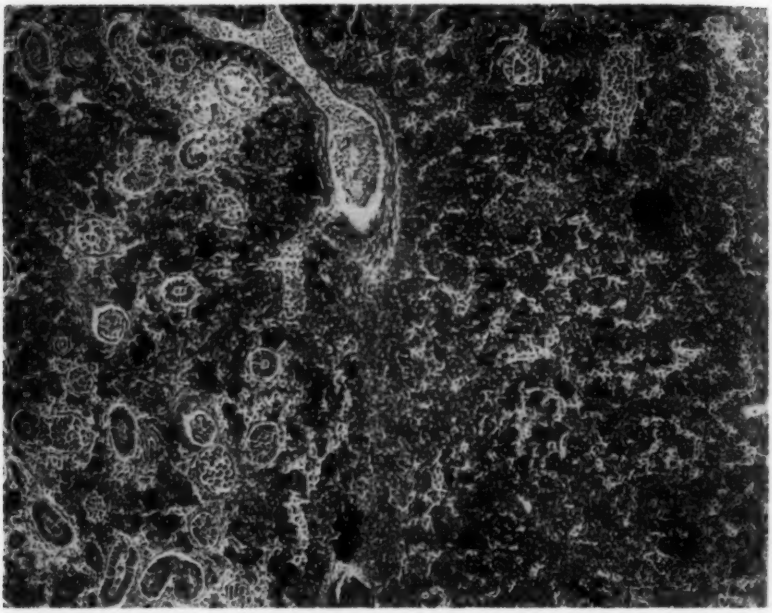

FIGURE 1. Photomicrograph of area of flounder kidney showing transition between anterior portion (right) containing few renal elements, and middle portion in which numerous glomeruli and tubules can be seen.

plasma. The regional distribution of mercury in the kidney is of interest. Both flounder and toadfish kidneys contain much lymphoid tissue in which the renal elements are imbedded, whereas the dogfish kidney on microscopic section shows tubular and glomerular structures surrounded by supporting tissue containing few or no lymphoid elements. Figure 1 shows a photomicrograph of the transition zone

TABLE III

Regional distribution of radiomercury in fish kidney. Combined data from fish injected with one milligram and 10 milligrams chlormerodrin mercury per kilogram and studied at time intervals up to 72 hours after injection. Results expressed as per cent of mean mercury concentration of tissue from the three regions of the kidney sampled

Species	No. of animals	Per cent of total mean mercury concentration in the kidney contributed by:		
		Anterior sample	Middle sample	Posterior sample
Flounder	16	9.0 ± 8.5*	48.5 ± 5.9	42.5 ± 5.6
Toadfish	15	26.6 ± 3.6	33.9 ± 5.1	39.6 ± 6.5
Dogfish	6	24.9 ± 7.6	35.4 ± 3.7	39.9 ± 8.2

* Standard deviation.

between the anterior and middle section of the flounder kidney. A few renal elements can be seen imbedded in the lymphoid cells in the anterior portion, and these elements can be seen in larger numbers in the more posterior portion of the section. The mercury concentration of the anterior block from which this particular section was made is one fifth that found in the more posterior section.

Table III represents a summary of the regional distribution of radiomercury in the kidneys of the species studied. The mean mercury concentration in anterior, medial, and posterior samples is assigned the value of 100%, and the proportion of the total contributed by the three regions is also expressed as per cent. It can be seen that in each species the lowest mercury concentration is found in the anterior portion, but that in the case of the flounder the difference is particularly striking.

Discussion

The species of marine vertebrates chosen for this study have nephrons which can be classified roughly into three types. The flounder can be considered to have kidneys with glomerular and proximal segmental function (Forster and Hong, 1958); the dogfish has a complex nephron, with glomerulus, proximal, and distal segment (Kempton, 1943). The toadfish, since the original description by Marshall (1929), has been considered to be both functionally and anatomically an aglomerular species with a nephron consisting of a proximal segment only.

The present experiments show clearly that the presence of a proximal segment is sufficient for a nephron to be capable of concentrating mercury. The high U/P ratio for mercury observed in the toadfish agrees with the findings in dogs of Borghgraef *et al.* (1956) that chlormerodrin mercury is excreted by means of a secretory mechanism. Subsequent "stop-flow" experiments have further localized this mechanism in the dog proximal tubular segment (Kessler *et al.*, 1958). Giebisch and Dorman (1958) have noted the selective accumulation of radiomercury administered as labeled chlormerodrin in the kidneys of carp, chicken, bullfrogs, and turtles, and have shown that in *Necturus maculosus*, a species in which the proximal and distal tubular segments are anatomically distinct, the proximal segment always contains a higher mercury concentration than does the distal. The observation of Bieter (1933) that mercuric chloride induces a diuresis in the anaesthetized toadfish also suggests that mercurial compounds act proximally. Unfortunately no analyses of toadfish urine for sodium and chloride are given with Bieter's experiments. The excretion of phenol red by the toadfish in the present experiments was also observed by Shannon (1938) and others.

The regional distribution of radiomercury noted in Table III deserves some comment. The injections of labelled chlormerodrin were made, for the most part, into the muscular portion of the tail, and since the kidneys of fishes are supplied largely by the renal portal circulation (Smith, 1953), it might be expected that the portion of the kidney closest to the injection site would contain the highest concentration of radiomercury. Such an explanation would agree with the results in both toadfish and dogfish, as seen in Table III, but in the case of the flounder the highest concentration of radioactivity is found in the medial portion of the kidney. It seems probable, therefore, that the low concentration of radiomercury in the anterior portion of the kidney in this species is a reflection of the small number of renal elements imbedded in the lymphoid tissue in this region (Fig. 1) and that

this represents an example of the strikingly higher accumulation of mercury by renal elements than by lymphoid tissue.

Mercurial diuresis in mammals is associated with an increased excretion of sodium and chloride ions. In the case of the marine fish, excretion of the sodium and chloride ions taken in via the mouth appears to be accomplished by the gills (Black, 1957). It is therefore interesting to note that these structures do not accumulate radiomercury under the present experimental conditions. Certain compounds of mercury are concentrated by the mammalian kidney to the same degree as is chlormerodrin mercury without affecting the excretion of water, sodium, or chloride (Kessler *et al.*, 1957). The present experiments with marine fish furnish evidence that the accumulation of mercury against a concentration gradient is a process which may occur in tissues not usually involved in sodium and chloride excretion. Studies in progress with the nephridia of *Phascolosoma gouldi* (Greif, 1957) are in agreement with this evidence.

Summary

1. Chlormerodrin labelled with radiomercury has been injected into marine fish with different types of nephrons. The kidneys of flounder (*Paralycthys dentatus*), with glomerulus and proximal tubular segment, of dog fish (*Mustelus canis*), with proximal, distal segment, and glomerulus, and toadfish (*Opsanus tau*), with proximal segment only, all concentrate mercury to a high degree.

2. High mercury U/P ratios in the toadfish indicate mercury excretion by a secretory mechanism. Excised flounder tubules will not concentrate phenol red if the animal has previously been injected with a toxic amount of chlormerodrin. Accumulation of mercury is also noted under certain conditions in the bile, especially in the dogfish, but is not seen in either stomach or gills.

3. Evidence is reviewed that in many animal species mercury accumulation is greatest in the proximal tubular segment.

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FURTHER EVIDENCE OF THE DESTRUCTION OF BIVALVE LARVAE BY BACTERIA[1]

ROBERT R. L. GUILLARD[2]

The Oyster Institute of North America, Inc.

Loosanoff (1954) has discussed the significance of large-scale culture of larval oysters and hard clams (*Venus mercenaria*) for experimental purposes and potential commercial use. It has been found that various organisms—the fungus *Sirolpidium*, for example—can destroy larvae in such cultures. However, evidence that bacteria are injurious has been largely circumstantial. Davis and Chanley (1956) showed that larval mortality was decreased at times by the use of various antibiotics. Walne (1956, 1958) found that antibiotics brought about increased spatfall of European oysters and parallel decrease of the bacterial population in his culture vessels.

Davis (1950, 1953), as part of feeding experiments, fed fourteen species of bacteria (including a mixture of *B. coli* and a bacteriophage) to oyster larvae. There was no evidence that any bacteria were of value as food. The four species used in the first experiments (*Vibrio marinofulvus, Micrococcus maripuniceus, Bacillus imomarinus,* and a red sulfur bacterium) were harmful at (unspecified) high concentrations, but not at low ones. However, three phytoflagellates studied produced the same results. In the second experiments, larvae fed bacteria (at unspecified concentrations) died within eleven days, while unfed controls grew slightly. Apparently mortality was not catastrophic. In this connection, observations by ZoBell and Feltham (1938) are significant. They found that adult mussels survived and grew when fed 10^8 to 10^9 washed bacterial/ml. once a day for nine months. Thirty-one clones were used. However, when a non-toxic peptone was added to water containing mussels, the animals died when the concentration of bacteria was only of the order of 10^5/ml. They suggested that metabolites in actively growing cultures were responsible, but it is possible that a different flora was selected by the addition of fresh nutrient solution. Brisou (1955) points out that *Pseudomonas*-like organisms are common in living bivalves. Takeuchi *et al.*, (1957) reported a high mortality of adult *Ostrea gigas* caused by or associated with a bacterium of the genus *Achromobacter*.

This report presents evidence that two clones of bacteria isolated from an infected hard clam (*Venus mercenaria*) larva destroyed healthy larvae, while other clones did not under similar experimental conditions. In the final experiments larvae were reared under aseptic conditions up to the time of exposure to known bacteria, thus excluding the possibility that contaminating microorganisms were the direct cause of death, while the bacteria were secondary invaders.

[1] This work was carried out at the Biological Laboratory, U. S. Fish and Wildlife Service, Milford, Connecticut.

[2] Present address: Woods Hole Oceanographic Institution, Woods Hole, Massachusetts.

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Materials and Methods

Isolation and growth of bacteria

Bacteria were seen "swarming" about moribund clam larvae in a laboratory culture having heavy mortality. One larva was transferred to a tube of sea water broth (essentially medium STP of Provasoli *et al.*, 1957), and the resulting mixed flora was subcultured daily. Pour and streak plates were made on the second day. Although most colonies appeared to be of two types, twelve obviously different clones were isolated. Ten other clones were isolated from contaminated algal cultures (*Monochrysis lutheri* or *Isochrysis galbana*) or from filters through which laboratory sea water was passed. Cultures of mixed bacteria were obtained by adding raw sea water to sterile broth.

Bacteria were grown at 28.5° C. for about 24 hours, with resulting concentrations of most clones of the order of 10^9/ml. STP broth was used in some of the first studies, but the medium was later standardized to ⅔ strength (Difco) nutrient broth made with sea water. In Experiment 2(2) bacteria were also grown in a clam broth made by autoclaving a minced adult hard clam in its own volume of sea water and decanting the supernatant. Suspensions of bacteria were diluted and samples killed and stained with I_2-KI and counted in a Petroff-Hausser chamber.

Filtrates of bacterial cultures (Experiment 2(3)) were prepared by drawing a few milliliters through sterile ultra-fine fritted glass filters. Filtrate was proved sterile by plating and inoculation into broth.

Bacteria were killed (Experiment 2(4)) by heating to 85° C. for five minutes. Cultures so heated did not grow upon subculture or streaking .

The concentration of bacteria to be used in the final experiments was estimated from measurement of concentrations in preliminary studies, which were of the order of 10^6/ml., and from observations made at various times during 1957–1958 of concentrations in apparently healthy larval clam and oyster cultures. In plate counts from 24–48-hour larval cultures (made on STP agar, ZoBell's No. 2216 agar (ZoBell, 1941), or ⅔ strength nutrient agar) bacterial concentrations were 10^5–10^6 per ml. However, counts of some of these cultures with a Petroff-Hausser chamber and dark field or phase contrast illumination yielded numbers of motile or clearly recognizable bacteria about an order of magnitude higher. (Concentrations in freshly changed cultures were of the order of 10^3–10^4 per ml., determined by plating.) Walne (1958) measured 10^4–10^5/ml. in 24-hour laboratory cultures of European oysters by plating on ZoBell's No. 2216 agar counted at 48 hours. It may be assumed that the actual concentrations were an order of magnitude higher.

Bacterial concentrations used in the final experiments were 10^6 to 10^7 per ml., provided from dense liquid cultures. Dilution made carryover of nutrients insignificant. (Because larvae had been observed to survive exposure to bacterial concentrations of the order of 10^8 per ml., this also was tried in Experiment 1. It was anticipated that larvae might be resistant to the bacteria because of pretreatment with antibiotics.)

Preliminary assays with non-aseptically-reared larvae

Bacterial cultures were assayed by adding 1- to 4-ml. aliquots yielding 10^6 to 10^7 bacteria per ml. to liter cultures of healthy larvae in freshly changed filtered sea water. (Methods of handling larvae are cited in a later section.) Larvae were maintained at a concentration of *ca.* 10/ml. at 24° C. and fed bacteria-free *Isochrysis galbana, ca.* 5×10^4/ml.

After 18–30 hours larvae were concentrated by screening and swirling and examined in a Sedgwick-Rafter cell with a compound microscope (× 150). Because the basic purpose of these assays was to screen bacterial cultures for obviously virulent strains, quantitative counts of mortality were not generally made. Rough quantitative counts were easily made by counting fewer than ten fields.

It was shown that the sterile nutrient broths were non-injurious to larvae at the concentrations used. This was done by adding sterile broth plus the antibiotic mixtures described below.

In each experiment there were two sets of control larvae; one received only food, the other received also an aliquot of sterile broth. In a few experiments bacteria developing in this latter beaker during the experimental period destroyed the larvae. These experiments were discarded even though the same flora might not have developed in the assay beakers.

Assay with aseptically-reared larvae

Straight-hinge clam larvae were obtained free of bacteria by allowing fertilized eggs to develop in solutions of antibiotics shown to be harmless to the animals. In the first experiment 50 mg./l. each of penicillin G (1625 units/mg.) and streptomycin sulfate were used. These concentrations were doubled in the second experiment and 50 mg./l. of chloramphenicol added. Oppenheimer (1955) showed that similar mixtures reduced viable bacteria in sea water to the order of a few per ml. in 24 hours. In our experiments, because adult clams were spawned in sterile sea water and larvae isolated with a micropipette after exposure to antibiotics, chances of contamination were negligible. No bacteria were detected by isolating larvae into sea water nutrient broth. Antibiotics carried over in the isolation technique were insufficient to prevent growth of bacteria.

Procedure

Sea water was autoclaved. Spawning dishes and screens were sterilized with ethanol and rinsed. Adult clams of known sex were washed in warm tap water, rinsed, and spawned by methods described or referred to by Davis (1953). Water was changed if clams did not spawn within a few hours. Fertilized eggs were passed through a 100-mesh screen to free them of feces and pseudofeces, then washed on a 325-mesh screen to free them of excess sperm and to concentrate them. After resuspension and counting, about 2000 eggs were added to 100 ml. of sterile filtered antibiotic mixture in 250-ml. Ehrlenmeyer flasks and kept at 24° C. Twenty-four or 36 hours later the fluid was poured into sterile Petri dishes and apparently healthy larvae caught with a pipette under a dissecting microscope.

Thirty to forty larvae were put into each of a series of 20 × 125-mm. screw-capped culture tubes containing 5 ml. of sterile sea water, and fed bacteria-free *Isochrysis galbana* at a concentration of 10^5/ml. (Control larvae in unchanged water in these tubes went to metamorphosis, but more slowly than those kept in containers as described by Davis and Guillard, 1958.) Aliquots of counted bacterial suspensions were added by pipette.

Motility of larvae could be observed through the test tube walls. At the end of the experiments, clams were again poured into a Petri dish and re-isolated onto a slide, where each was examined with a compound microscope using dark field and phase contrast illumination as necessary.

Experimental

Assay with non-aseptically-reared larvae

Mortality of larvae exposed for 18–30 hours to the mixed culture derived from a moribund clam and to the five succeeding subcultures from it was greater than 90%. "Swarming" bacteria were numerous in moribund animals and were seen swimming freely. No comparable mortality was observed in five trials involving the ten clones derived from contaminated algal cultures or the mixed bacteria resulting from the enrichment of sea water. (Not all clones were included in each of the trials.) Larvae were able to survive in concentrations as high as 10^8/ml. of some of these clones, though they developed abnormally in the higher concentrations. Mortality was negligible in both sets of controls in all these trials.

Two assays were made of the twelve clones isolated from the moribund clam. Ten of these caused no significant mortality, but the other two produced mortality comparable to that of the mixture from which they were derived. These clones, designated 6–1 and 13–1, were the two colony types predominating on agar plates of the mixed culture. Both are gram-negative, non-sporogenous, polar monotrichous rods about 0.75 × 1–2.5 microns in size. Both are halophilic to some extent and have not become adapted to growth on media without NaCl or sea water. The temperature optimum of 6–1 is between 35° and 40° C., while that of 13–1 lies between 25° and 32.5° C. Dr. Einar Liefson of Loyola University has undertaken further study of both strains. He has assigned 6–1 to the genus *Vibrio* and 13–1 to *Pseudomonas*. Some criteria are given in Hugh and Leifson (1953).

Experiment showed that neither 6–1, 13–1, nor the mixture from which they came could injure larvae in the presence of antibiotics. Duplicate larval cultures were inoculated with *ca.* 10^7 bacteria per ml.; to one set was added also 50 mg./l. each of penicillin G and streptomycin sulfate. Animals exposed to bacteria alone were destroyed in 24 hours, while those treated with antibiotics also were indistinguishable from controls exposed to neither or to antibiotics alone. This was done twice with each bacterial culture.

Experiments with aseptically-reared larvae

Experiment 1. This was undertaken primarily to test the method and to determine if bacteria at the concentrations used would kill larvae previously exposed to antibiotics. The experiment proper consisted of eight tubes, as follows:

1. controls, sea water
2. controls, nutrient broth
3. clone 13–1, 10^6/ml.
4. clone 13–1, 10^7/ml.
5. clone 13–1, 10^8/ml.
6. clone 6–1, 10^6/ml.
7. clone 6–1, 10^7/ml.
8. clone 6–1, 10^8/ml.

There were also five tubes in which larvae were grown to metamorphosis.

Examination through the tube walls showed that most larvae were killed in a day in the highest concentration of each clone. The other tubes were examined on the fourth day. Results are summarized in Table I. Larvae exposed to 10^7/ml.

TABLE I

Mortality of clam larvae caused by three concentrations of clones 6–1 and 13–1 (larvae initially 115–120 microns in size)

Clone	Bacteria per ml.	Time	No. of larvae moribund	No. of larvae recovered	% of larvae moribund	Notes
13–1	10^8	1 day	26	27 of 30	96	vela disintegrating
13–1	10^6	4 days	3	29 of 30	10	none over 135 μ
13–1	10^7	4 days	38	40 of 40	95	none over 135 μ
6–1	10^8	1 day	27	32 of 35	84	none over 125 μ
6–1	10^6	4 days	22	29 of 30	76	largest were 160 μ; dead larvae were 120–145 μ
6–1	10^7	4 days	11	11 of ?	100	vela swollen; none over 135 μ
Control	none	4 days	0	30	0	135–160 μ*

* Another group of larvae from the same spawning was kept in polyethylene containers, fed every day and changed every other day. The largest of these larvae were 190 μ.

of either clone were 95%–100% destroyed by the fourth day. However, 10^6/ml. of 13–1 caused only 10% mortality, while a like number of 6–1 killed 76%. No control animals died.

Bacteria were not counted during or after the experiment. Judging subjectively, there were not as many in the medium at the end as might have expected on the basis of the number added. Some of the moribund larvae were surrounded by "swarming" bacteria.

Experiment 2. The objectives were:

1. To compare the effects of bacteria on larvae kept at three different temperatures; 20, 25, and 30° C. The observation that clones 6–1 and 13–1 grew better at high temperatures than did most other clones isolated suggested this portion of the experiment.

2. To compare the effects on larvae of equal numbers of bacteria grown on two different media—the usual nutrient broth and clam broth. The possibility that bacteria maintained on clam broth might remain more virulent than those maintained on nutrient broth prompted this portion of the experiments.

3. To observe larvae exposed to sterile filtrate of bacterial cultures.

4. To observe larvae exposed to dead bacteria plus their culture broth. Experiments 3 and 4 were to confirm that only living bacteria kill larvae, as suggested by the antibiotic experiment of Section 1. The experiment was carried out in 20 tubes, as follows:

Tube	Temperature °C.
1. Control, food only	25
2. 6–1, 10^7/ml. (broth-grown)	25
3. 13–1, 10^7/ml. (broth-grown)	25
4. Control, food only	20
5. 6–1, 10^7/ml. (broth-grown)	20
6. 13–1, 10^7/ml. (broth-grown)	20
7. Control, food only	30
8. 6–1, 10^7/ml. (broth-grown)	30
9. 13–1, 10^7/ml. (broth-grown)	30
10. 6–1, grown in clam broth, 10^6/ml.	25
11. 6–1, grown in clam broth, 10^7/ml.	25
12. 13–1, grown in clam broth, 10^6/ml.	25
13. 13–1, grown in clam broth, 10^7/ml.	25
14. 6–1 filtrate, 1 ml. (equivalent to $1\,6 \times 10^9$ bacteria/ml.)	25
15. 13–1 filtrate, 1 ml. (equivalent to 3.2×10^9 bacteria/ml.)	25
16. Heat-killed 6–1 (8×10^8/ml.)	25
17. Heat-killed 13–1 (8×10^8/ml.)	25
18. Larvae in broth alone as sterility check	25
19. Larvae in broth alone as sterility check	25
20. Larvae in broth alone as sterility check	25

Larvae were examined on the fifth day. In Table II and Table III, respectively, are gathered data pertinent to the temperature portion of the experiment and comparison of the effects of bacteria grown on different media.

Mortality was relatively independent of the temperature at which larvae were kept: 73% to 76% of the animals exposed to clone 6–1 were dead, as were 30% to 48% of those exposed to clone 13–1. Maximum mortality in controls was 7%. It should be noted that growth in the 30° C. controls was poor and that food organisms settled in the tubes at this temperature. (Both 20° and 30° controls were bacteria-free at the end of the experiment.)

From Tables I and II it can be seen that there were no consistent differences in mortality caused by bacteria grown on the two different media. Greatly increased virulence would have been evidenced by early heavy mortality easily visible through the culture tube walls. Small but significant differences would not be detected by an experiment such as this.

The hypothesis that only living bacteria destroy larvae was confirmed by the findings in tubes 14 through 17, in which larvae were exposed to filtrate corresponding to more than 10^9 bacteria per ml. or to 8×10^8 dead bacteria in their broth. Of 120 larvae, only two were dead, one in tube No. 15 and one in No. 17. How-

TABLE II

Comparison of mortality of larvae exposed to bacteria and maintained at three different temperatures for five days (concentration of bacteria 10^7/ml. Larvae initially 105–110 microns in size)

Clone	Temp. °C.	% larvae moribund	Average size		Maximum size	
			Living	Moribund	Living	Moribund
6–1	20	73	125	120	145	125
6–1	25	75	130	115	150	125
6–1	30	76	120	120	130	120
13–1	20	30	150	125	165	150
13–1	25	46	145	125	160	140
13–1	30	48	135	120	185	135
None	20	7	145	—	165	—
None	25	0	140	—	165	—
None	30	3	125	—	140	—

ever, larvae grew scarcely at all. They were initially 105–110 microns in size, and increased only to 110–120 microns. (The larger animals seen in control tubes were 165 microns in size, see Table III.) Moreover, larvae exposed to filtrate or dead bacteria were not feeding and were emaciated. This was probably due to the high concentration of metabolites. In the preliminary assays (Section 1) in which larvae were exposed to living bacteria together with antibiotics, those animals exposed to both bacteria and antibiotics were indistinguishable from controls, but the amount of inoculum in this case corresponded only to 10^6–10^7 bacteria/ml. rather than to 10^9/ml.

In both experiments 1 and 2, moribund larvae injured by clone 6–1 differed in appearance from those injured by 13–1. The former often had vela so distended that they exceeded the bodies of the larvae in length. Some vela appeared to be decomposing and occasionally they detached from the rest of the clam. The bodies of larvae often had striations visible going from the hinge towards the velum.

Clams injured by 13–1 usually had vela that were frayed or ragged, with cilia largely or entirely missing. The bodies were emaciated and granular in appearance. Some seemed to be disintegrating from the hinge side towards the velum. Mori-

TABLE III

Comparison of the mortality at 5 days of larvae exposed to bacteria grown in (a) 2/3 strength nutrient broth and (b) clam broth (larvae initially 105–110 microns in size)

Clone	Medium	Bacteria per ml.	% larvae moribund	Average size		Maximum size	
				Alive	Moribund	Alive	Moribund
6–1	nutrient	10^7	75	130	115	150	125
6–1	clam	10^6	40	135	125	150	135
6–1	clam	10^7	97	120	120	120	120
13–1	nutrient	10^7	46	145	125	160	140
13–1	clam	10^6	59	135	120	140	135
13–1	clam	10^7	48	135	120	150	135
Controls		none	none	140	—	165	—

bund larvae in laboratory cultures often fit one of these descriptions, but it is not known if the appearance is in fact correlated with an infecting bacterium.

Discussion

While the mechanism by which clones 6–1 and 13–1 destroy larvae was not studied, evidence available favors the hypothesis that it is by invasion or at least contact rather than by an exotoxin liberated into the medium. The fact that larvae withstood relatively large amounts of glass-filtered, heat-killed, or antibiotic-treated bacterial culture shows that an exotoxin, to be the sole agent, would have also to be extraordinarily unstable. Further, an exotoxin would be expected to have a relatively uniform influence on animals exposed to it, so that larvae would be more or less uniform in size. In fact, however, larvae exposed to bacteria varied considerably in size (Tables II and III), rather in keeping with the hypothesis that they continued to grow until invaded. Finally, there are observations that mortality in large cultures often followed the pattern of an epizootic, and that bacteria were frequently seen swarming in dying or dead larvae.

It is not implied that bacterial metabolites are without influence on larval growth or development. Indeed, the experiment showed that high concentrations stopped growth entirely. It has also been observed that bacterial contamination of algal food cultures sometimes caused abrupt decrease in larval growth rate without immediate extensive mortality. The addition of cultures of bacteria (other than 6–1 or 13–1) often did the same. This depressant effect may well be due to exotoxins.

Strains 13–1 and 6–1 were far more virulent than other bacteria tested and clearly are a hazard to larvae under laboratory conditions. Possibly they were favored by conditions in the larval cultures and finally dominated the flora, at which point the "disease" became obvious. The observation that both strains grow well at temperatures over 30° C., which is relatively uncommon in marine bacteria found locally, supports this idea. At present it is not possible to tell if these bacteria destroy larvae in nature, where both bacteria and larvae are usually less concentrated than they are in cultures. If the "disease" occurs in nature, one would expect to find it under conditions of high temperature and restricted water exchange.

It should be mentioned that the use of antibiotics to control bacteria in larval cultures is apparently more effective when the water supply is changed regularly and two or more antibiotics are used alternately. Probably this prevents the development of a resistant flora. Animals other than bivalves are also benefited; J. Hanks (personal communication) found that larvae of the gastropods *Polinices duplicata* and *P. heros* grew better when penicillin and streptomycin were used in this way. It must be emphasized, however, that the same antibiotics will not prevent growth of injurious bacteria in algal cultures used as food. If impure algal cultures must be used to raise larvae, the algae should obviously be kept at the lowest temperature allowing reasonable growth.

Summary

1. Twelve strains of bacteria were isolated from a moribund *Venus mercenaria* larva in a laboratory culture. These, ten other clones, and mixed bacteria from

sea water were assayed by adding broth culture yielding 10^6–10^7 cells/ml. to beaker cultures of healthy clam larvae. Only the mixed bacterial culture from the moribund larva and two of the 12 strains isolated from it caused extensive mortality. One of the virulent clones (6–1) is a species of *Vibrio*, the other (13–1) is a *Pseudomonas* species.

2. Larvae exposed to virulent bacteria and simultaneously treated with antibiotics were as healthy as controls, showing that active bacteria were necessary to destroy larvae and that metabolites in the bacterial inoculum were not harmful to larvae.

3. Larvae were grown free of contaminating micro-organisms by allowing washed eggs to develop in antibiotic solutions and then isolating straight-hinge larvae by pipette. Either virulent clone (10^6–10^7/ml.) destroyed 10–100% of such larvae. However, exposing the animals to large amounts of glass-filtered or heated broth in which bacteria had been grown (corresponding to *ca.* 10^9 bacteria/ml.) caused no mortality, but retarded growth.

4. Mortality caused by clones 6–1 and 13–1 in groups of clams kept at 20°, 25°, and 30° C. did not vary significantly. However, both virulent clones grow well at 30° C. and higher; thus high temperatures in laboratory larval cultures favor these strains.

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NEUROSECRETORY CELLS IN GANGLIA OF THE ROACH, BLABERUS CRANIIFER [1]

SEMAHAT GELDIAY

Department of Zoology, University of Ankara, Turkey, and Columbia University New York 27, N. Y.

Neurosecretory cells have been described in many groups of insects (Scharrer and Scharrer, 1954). Most observations have been made upon brain, subesophageal and frontal ganglia, and corpora cardiaca. There is relatively little information concerning neurosecretion by cells within the thoracic and abdominal ganglia of insects. Day (1940) described neurosecretory cells in the abdominal ganglia of Lepidoptera, and Köpf (1957) found neurosecretory cells in the thoracoabdominal ganglion of *Drosophila*. E. Thomsen (1954) found no evidence that neurosecretory materials were accumulating on either side of ligatures of abdominal connectives in *Calliphora* and concluded that these connectives did not carry neurosecretory products.

During an investigation of the functional roles of the neurosecretory materials accumulated within the corpora cardiaca of the roach, *Blaberus* (Özbas and Hodgson, 1958), the search for appropriate controls, *i.e.*, tissues which would contain only non-secretory neurons, prompted an examination of the thoracic and abdominal ganglia of this species. It immediately became apparent that neurosecretory cells of more than a single type were present in all of these ganglia. Since the existence of neurosecretory cells had not been previously reported within the thoracic or abdominal ganglia of Blattaria, and since this order of insects is commonly utilized in experiments on neurosecretion, it appeared worthwhile to make a detailed study of the neurosecretory cells at these hitherto unstudied loci.

The object of the present report is to describe the types of neurosecretory cells present in the thoracic and abdominal ganglia of *Blaberus*, and in so far as it is possible, to trace the movements of secretions produced by these cells, using evidence from ligation and histological studies. The relationships of the neurosecretory cells within the ganglia to secretory cells located elsewhere within the nervous system are also considered.

Materials and Methods

The last three larval instars and adults of *Blaberus craniifer* were studied. No significant differences correlated with sex or developmental stages were found among specimens of this group. Only adults were used in ligation experiments because their larger size afforded technical advantages for the operations.

Two fixatives and two stains were used on each type of preparation, so that by

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comparing the results of the various methods it was possible to rule out artifacts produced by any one fixative or stain. Either Bouin or Helly fixatives were used. The stains were the chrome hematoxylin phloxin stain of Gomori (1941), hereafter designated CHP, or the aldehyde fuchsin stain (Gomori, 1950) as modified by Halmi (1952) and Dawson (1953), hereafter designated AF. All sections were cut 5 microns in thickness.

Ligatures were prepared from Clark's Size A black nylon thread. A single strand was teased from this thread and tied around one of the two connectives which pass between the ganglia. The operations were performed through incisions in the ventral sides of unanesthetized animals, and the ligature sites were varied in different animals so that blocks of connectives between each of the thoracic and abdominal ganglia were represented in the total series of 44 operated animals. The operated roaches were maintained in separate pint jars and fed dogfood, fruit, and water, this being the same diet given the standard laboratory colony of *Blaberus*. The animals were sacrificed 5 to 40 days after the operation, the nerve cords dissected out, and histological preparations made by the methods outlined above.

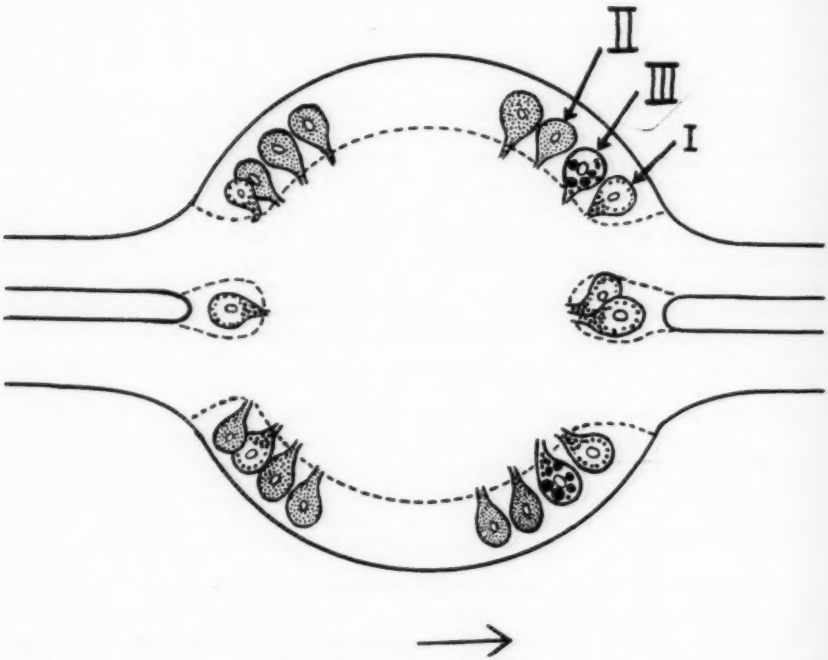

FIGURE 1. Diagrammatic representation of a frontal section through the center of a thoracic ganglion, showing arrangement of neurosecretory cells. The dotted lines separate the peripheral areas which are occupied by cell bodies from the central neuropile mass. Roman numerals designate types of neurosecretory cells—see text. The arrow under the diagram points anteriorly, toward the animal's head.

Results

1. *Neurosecretory cells within the ganglia*

The thoracic and abdominal ganglia were found to contain three types of neurosecretory cells, as shown diagrammatically in Figure 1, and in the photographs A, B, and C of Figure 2. These cells can be differentiated from ordinary neurons by their size, staining characteristics, and the presence of characteristic granules or droplets within their cell bodies and axons. The cell bodies of all three types of neurosecretory cells measure 40 to 60 microns in their longest diameters, 35 to 50 microns in their shortest diameters, and have ellipsoid nuclei measuring 15 to 18 microns in their shortest diameters and 18 to 22 microns in their greatest diameters. The nucleoli are also conspicuous in many of the stained neurosecretory cells. The distinguishing characteristics of the three types of cells are described in the following paragraphs.

Type I (see Fig. 2A). The cell bodies and axon hillocks of these cells contain granules which stain deep purple in AF and dark blue in CHP. There are 5 to 10 such cells in each thoracic and abdominal ganglion, and they are located in the outer portion of the ganglion near the origin of the connectives (see Fig. 1).

Type II (see Fig. 2B). These neurosecretory cells have very small granules distributed uniformly throughout the cell body. The granules stain green with AF and red with CHP. These are the most common neurosecretory cells in thoracic and abdominal ganglia, and there are at least several dozen cells of this type distributed generally throughout the periphery of each ganglion (see Fig. 1).

Type III (see Fig. 2C). Only two neurosecretory cells of this type appear to be located within any one ganglion. They are seen only after Helly fixation and never after use of Bouin solution. The two cells are found in the anterior lateral part of the ganglion, one cell on each side of the ganglion (see Fig. 1). They are characterized by the presence of large droplets which stain orange with AF. These droplets have diameters of 3 to 11 microns. They resemble the "colloid droplets" described by E. Scharrer (1941) within the cells of the preoptic nucleus of the fish, *Fundulus*. There was no indication of cycles of formation or alteration of the droplets in the roach sections such as reported in *Fundulus*.

2. *Observations on normal axons and connectives*

Neurosecretory materials from all three types of neurosecretory cells were observed within axons extending into connectives between ganglia. Small granules, such as found in type I cells, are usually found in no more than one or two axons per section of connective (Fig. 2F). These granules fill the axons, where found, and they appear to move in definite oriented chains.

Secretory material from type II cells is best seen in axons after staining with CHP. This material is the most abundant in axons, and is seen in practically every section of connectives between ganglia. Orange material, presumably from type III cells, was seen in axons of only two animals. The rarity of this material in axons may reasonably be explained by the rarity of the type III cells. When observed in axons, the orange material was not in the form of round droplets, but had the form of elongated rods.

Many glia cells were seen between axons in the connectives and also in the ganglia. These cells were described by B. Scharrer (1939) in the brain, sub-

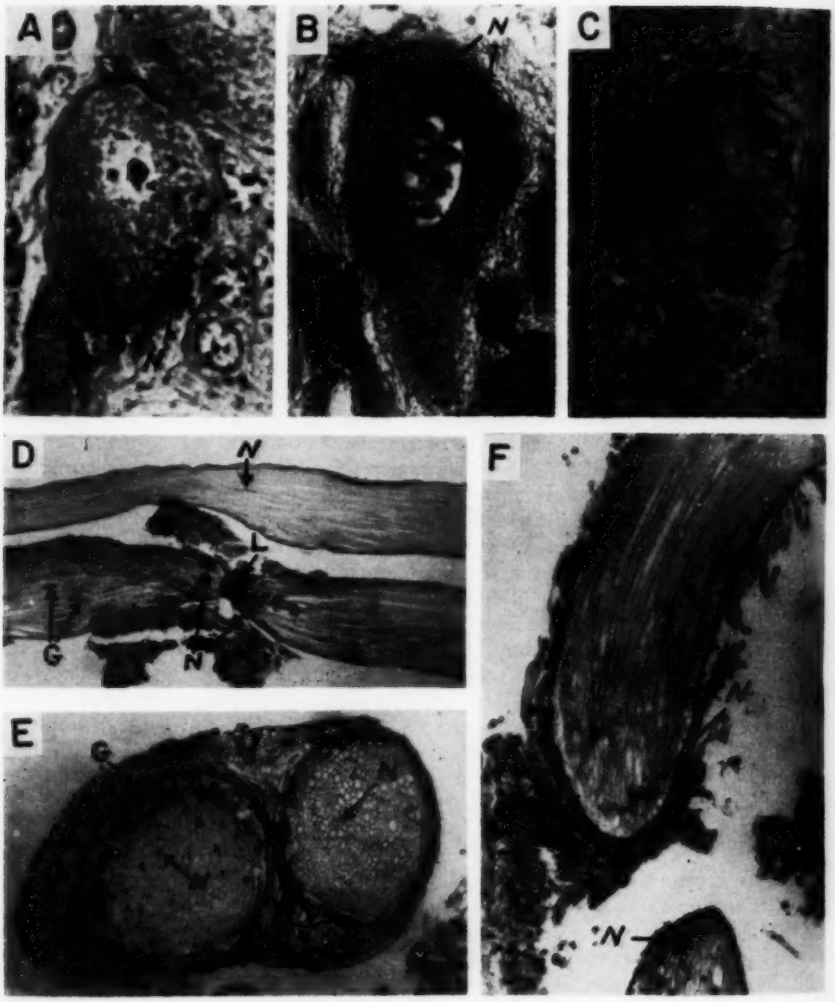

FIGURE 2. Histological preparations. A—type I cell from metathoracic ganglion of adult male (Helly fixation with AF), 1080 ×; B—type II cell from prothoracic ganglion of adult male (Bouin fixation with CHP), 1080 ×; C—type III cell from mesothoracic ganglion of adult female (Helly fixation with AF), 1080 ×; D—frontal section through connective between subesophageal ganglion and prothoracic ganglion (adult male, ligation on animal's right side). Seven days after operation. (Helly fixation with AF), 72 ×; E—cross-section anterior to ligation in left connective between prothoracic and mesothoracic ganglia, control side on right. ♀ adult 20 days after operation (Helly fixation with AF), 144 ×; F—frontal section of left side connective ligated and broken between subesophageal and prothoracic ganglia, ♀ adult, 10 days after operation (Helly with AF). N—neurosecretory materials. G—glia cells. L—ligature.

esophageal ganglion, and connectives of *Periplaneta*. The glia cells are spindle-shaped, have elongated nuclei, and contain gliosomes staining deep purple with AF and deep blue with CHP. In some cases, particularly when the nucleus of the glia cell is not seen, the glia cells might be confused with neurosecretory particles inside connectives, but the latter are in much longer chains and are larger particles than the gliosomes. Roles as supporting cells have been ascribed to the glia cells (B. Scharrer, 1939).

3. *Ligation experiments*

Since ligation of nerves containing neurosecretory axons has been shown to result in accumulations of neurosecretory materials on those sides of the ligations where the neurosecretory materials originate (E. Thomsen, 1954), ligation experiments were used to determine the directions in which the neurosecretory materials were moving. The ligation technique has been described above. In a total of 44 ligation operations, 4 animals died between 10 hours and 12 days after the operation. In 24 cases, the ligated connective was found broken and the nylon thread found in the hemolymph in the vicinity of the operation site. In 16 cases, the connective was not broken and the thread was found still in place (see Fig. 2D).

Whether or not the connective was broken, portions of connective both anterior and posterior to the break or ligation were swollen, as compared to the corresponding unoperated connectives (see Figs. 2D, 2E). The operated connectives, as studied in histological sections, differed from connectives on the control sides in several other ways as well: (a) more glia cells were present on the experimental side, not only in the broken or ligated tips of the connectives, but along their entire lengths (see Fig. 2D); (b) neurosecretory products of types I and II were accumulated in axons on both sides of the breaks, not only localized at the broken or ligated areas, but in elongated masses accumulated within axons on both sides of the operation (Figs. 2D, 2F); (c) no accumulation of type III neurosecretion was seen, doubtless because of its rarity even in the normal connectives.

It is therefore possible to conclude from the ligation experiments that neurosecretory materials from neurosecretory cell types I and II within the thoracic and abdominal ganglia pass in both anterior and posterior directions through the interganglionic connectives. Secretions from type III cells also pass into the connectives but are present in such limited quantities that their movements have not been intercepted in these ligation experiments.

Discussion

With the steadily increasing numbers of neurosecretory cells being described, particularly as new histological procedures are adopted, it is obviously desirable to prevent unnecessary duplication of terminology. Consequently, a more extended comparison of the neurosecretory cells described here with types already described seems appropriate. The most exact resemblance is between the cells which are here designated type II and those designated type B by Nayar (1955) and Köpf (1957). These cells also appear identical to ones staining pink with CHP in the pars intercerebralis of *Blaberus* (Özbas and Hodgson, 1958). Cells of similar staining characteristics have also been seen in the pars intercerebralis of Diptera and Hymenoptera (E. Thomsen, 1954; M. Thomsen, 1954). The uniform dis-

tribution of granules within the cell bodies of all these neurosecretory cells and their staining characteristics strongly suggests that they might properly be considered as homologous neurosecretory cells in several groups of insects.

Type I cells are most nearly comparable with, but not identical to, the cells designated type A by Nayar (1955) and Köpf (1957), and previously seen by other workers in the pars intercerebralis of many insects (Scharrer and Scharrer, 1954; De Lerma, 1956; Dupont-Raabe, 1956; Özbas, 1957). The similarities between type I and type A cells consist of the shapes of the cells and their staining properties with CHP. Nayar describes cytoplasm filled with neurosecretory material in type A cells, but in the preparations of thoracic and abdominal ganglia, the neurosecretory material is always near the edge of the cell body. This arrangement of granules within the cell body resembles that described by Scharrer (1955) in the "castration cells" of the subesophageal ganglia of *Leucophaea*. Nayar noted no selective staining of type A cells with AF. Although the AF stain used here is a later modification of the AF staining technique used by Nayar, typical A cells were seen in the pars intercerebralis of *Blaberus*. Cells of identical staining characteristics, but having cyclic secretory activity, were found in the subesophageal ganglion of *Blaberus* during the present study; these cells have been described earlier by B. Scharrer (1941). The distinction between type I and Type A cells must, therefore, be a real one and not a dissimilarity caused by differences of staining technique.

The type III cells described in this report have not been previously described in any insect. Although the droplets which they contain resemble the "colloid droplets" found in certain neurosecretory cells of *Fundulus* (E. Scharrer, 1941), this resemblance cannot be interpreted as implying identity of cell types in such widely divergent groups of animals.

The conclusions from the ligation experiments are contrary to those drawn from ligation experiments on abdominal cords of *Calliphora* by E. Thomsen (1954). However, the *Calliphora* ligations were performed only as controls for other experiments and the results were not studied histologically. Since few axons in the abdominal connectives carry neurosecretory materials, it is not surprising that Thomsen did not find accumulations of neurosecretions in the ligated abdominal connectives which would be seen by inspection of the whole live connectives. The small quantity of neurosecretory material which would be detected in unligated abdominal connectives probably also explains why none was detected when observations were made upon whole central nerve cords using darkfield illumination (M. Thomsen, 1954).

Attempts to detect effects of neurosecretory products from thoracic and abdominal ganglia upon spontaneous electrical activity of *Blaberus* nerve cords *in vitro*, similar to the effects of neurosecretion from the corpora cardiaca (Özbas and Hodgson, 1958), were unsuccessful. This may be due to the small amounts of neurosecretory materials present even in whole thoracic and abdominal ganglia. Moreover, the most abundant type of neurosecretory cells in these ganglia, type II, is relatively scarce in the pars intercerebralis, and all evidence seems to indicate that it is not their secretion which accounts for the effects of corpus cardiacum extracts upon spontaneous nerve activity (Özbas and Hodgson, 1958).

What may be postulated, then, as a normal function of the neurosecretions

produced in the ganglia and passing anteriorly and posteriorly through the interganglionic connectives? Although the histological sections were always examined with such a possibility in mind, no clues were found in the form of any areas where neurosecretions from the ganglia were normally being accumulated. It must be assumed that the sites of release of the neurosecretory products are widely scattered throughout the central nervous system, regardless of where their effects are ultimately exerted. Concerning this problem of the normal functions of the neurosecretory cells here described, the present observations can really only be an introduction.

This work was done in the Department of Zoology, Columbia University. The author wishes to express her thanks to Professor E. S. Hodgson for his help and encouragement during the course of the investigation and for his help in preparing the manuscript.

Summary

1. Three types of neurosecretory cells are found within the thoracic abdominal ganglia of the roach *Blaberus craniifer*. These three cell types may be characterized as follows: type I—contains granules staining deep purple with aldehyde fuchsin, the granules being distributed around the periphery of the cell body and in the axon hillock; type II—the most common neurosecretory cell in the ganglia, contains very small granules staining red with chrome hematoxylin and phloxin, the granules being distributed throughout the cell body; type III—the rarest of the neurosecretory cells observed within the ganglia, contains large droplets staining orange with aldehyde fuchsin. The possible identities or homologies of these cell types with others previously described in insects are discussed.

2. Secretory products from all three types of neurosecretory cells have been found within axons in the connectives between ganglia.

3. Ligation of the connectives between ganglia reveals that at least neurosecretory products of cell types I and II move in both anterior and posterior directions from the ganglia in which they are produced. Neurosecretion from cells of type III is very rarely seen in connectives and, consequently, its direction of movement could not be established.

4. No areas of accumulation for any of the neurosecretions were found within the central nerve cords, and the normal functions of these secretions are not known.

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EXPERIMENTALLY INDUCED RELEASE OF NEUROSECRETORY MATERIALS FROM ROACH CORPORA CARDIACA[1]

E. S. HODGSON AND S. GELDIAY

Departments of Zoology, Columbia University, New York 27, N. Y., and University of Ankara, Turkey

Although the pars intercerebralis-corpus cardiacum system of insects has been extensively used for experimental analysis of neurosecretion (B. Scharrer, 1954; Wigglesworth, 1954), there are relatively few data to indicate the conditions under which this system normally releases neurosecretory substances during the life of the animal. Variations in the amount of neurosecretory materials within the corpora cardiaca are known to occur according to the age and the physiological conditions of insects (B. Scharrer, 1952; Wigglesworth, 1954). Variations in the potencies of corpora cardiaca extracts in affecting central nervous activity, as tested by the method of Özbas and Hodgson (1958), have recently suggested that corpora cardiaca from roaches which have been extensively handled or subjected to prolonged surgical procedures contain significantly less neurosecretory material than normal. An analogy with the secretion of adrenalin during the response of mammals to stress situations is further suggested by the isolation from corpora cardiaca of a substance with adrenalin-like effects upon roach and frog hearts (Cameron, 1953; Unger, 1957).

The present experiments were designed to test the hypothesis that neurosecretory materials are released from the corpora cardiaca of the roach when the animal is hyperactive or experiences conditions resembling those which produce symptoms of stress in mammals. Another objective of this work has been to determine whether experimentally induced "stress" conditions produce histologically detectable changes in the neurosecretory cells of the pars intercerebralis or other changes within the brain.

Materials and Methods

The roach *Blaberus craniifer* was the experimental animal. Each experimental group consisted of adult females which had undergone their last molt on the same day. This selection was made because some males lack one of the neurosecretory products always observed in corpora cardiaca of adult females of this species (Özbas and Hodgson, 1958), and in order to have the experimental animals as uniform as possible.

Experimental treatments of the roaches consisted of administering electrical shocks to the animals or forcing them to be hyperactive. Ten-volt electrical shocks of 5 milliseconds duration each were administered from an electronic square wave stimulator at the rate of twenty shocks per second. Steel electrodes (size 0 insect pins) were inserted into bilaterally symmetrical positions in the roach's head,

[1] This investigation was aided by a U. S. Public Health Service Grant (No. E-2271) to Dr. Edward S. Hodgson.

near the medial margins of the compound eyes, or else one electrode was inserted into the right lateral portion of the third abdominal segment and the other electrode inserted into the left lateral portion of the fifth abdominal segment. The shocks thus administered represent approximately the minimum amplitude, duration, and frequency of electrical stimulation to which these roaches gave behavioral responses consisting of abdominal movements and movements of the appendages. Locomotion was prevented during the administration of shocks by pinning each roach through the edges of its pronotum to a dissecting board. Some control animals, hereafter designated C1, were pinned to the board without electrodes, while others, designated C2, were pinned and also had electrodes in the head and abdomen but received no shocks.

Sustained hyperactivity of *Blaberus* was produced by placing the roach within a glass jar and giving the jar a quick shake so that the roach was turned upside down. This posture invariably caused the roach to execute violent leg and wing movements until it had turned itself over, whereupon the jar was immediately shaken again so as to invert the roach and initiate its struggles again. Each animal's activity was sustained by repetition of this treatment throughout the desired length of time.

Control and experimental animals were killed by decapitation at various intervals after treatment, and the entire head (including the corpora cardiaca) of each roach was immediately fixed with either Bouin's or Helly's solution, as specified below. Five-micron sections were cut and stained with either the chrome hematoxylin phloxin stain of Gomori (1941), hereafter designated CHP, or the aldehyde fuchsin stain (Gomori, 1950) as modified by Halmi (1952) and Dawson (1953), designated AF. Since the numbers of animals in each experimental group varied according to the numbers of synchronously molting females available, and their treatments varied according to the information sought, each experimental series will be described separately.

Results

Series I consisted of 6 roaches tested 31 days after molting. Two of these were used as controls (C1 and C2), treated in the manner described above. Both controls were pinned to the dissecting board for 15 minutes and sacrificed immediately thereafter. Animal No. 3 had electrodes in its head only, and received shocks for one minute; animal No. 4 also had electrodes in the head only, but received shocks for 15 minutes; No. 5 had electrodes in the abdomen and received shocks for 15 minutes; No. 6 had one set of electrodes in the head and another set of electrodes in the abdomen, and it received shocks through both sets of electrodes for one hour. All of the animals in this series were fixed in Bouin's solution within one minute after the end of treatment, and the sections were stained with CHP.

The sections revealed marked differences in the amounts of neurosecretory materials within the corpora cardiaca of these animals. The corpora cardiaca of the controls (both C1 and C2) had large amounts of neurosecretory materials staining dark blue and pink. These neurosecretory materials were distributed throughout the central parts as well as the peripheral regions of the corpora cardiaca. Less of both pink and blue staining materials could be seen in sections from animals No. 3, but the most striking results were obtained in the cases of

animals 4, 5, and 6. These three roaches had very little of either the pink or blue staining materials remaining in the corpora cardiaca, and most of the traces were found in the peripheral regions of the glands, especially near the aorta. These results indicate a significant loss of both kinds of neurosecretory materials from the corpora cardiaca during the experimental shock treatments lasting 15 minutes or longer.

Since there is some variation in the initial amounts of neurosecretory materials within the corpora cardiaca of different animals, and even in the amounts observed within adjacent 5-micron sections from the same gland, the interpretation of an apparent partial decrease in neurosecretory content of glands from one animal alone, such as No. 3 from this series, would be questionable. The differences between the two controls and the animals receiving shock treatments for 15 minutes or more are very large, however, not only in the total amounts of neurosecretory materials within the glands, but also in the distribution of the materials as described above. To this evidence must be added the results from the other experimental series also.

Series II consisted of 6 animals tested 49 days after molting. The tests were designed to check the reproducibility of the experimentally induced decrease of neurosecretory substances found in Series I, and to determine the rate of restoration of the depleted substances within the corpora cardiaca. Two control animals (C1 and C2) were treated exactly as those in Series I. Each of the other 4 animals received shocks for 15 minutes through electrodes in the abdomen, thus duplicating the treatment given animal No. 5 of Series I. The 4 experimental animals in Series II were sacrificed at the following intervals after the end of their shock treatments: one minute, one hour, 6 hours, and 24 hours.

Since total amounts of neurosecretory materials were of concern in this series, the AF stain, following Helly's fixative, was chosen as the most convenient way of analyzing the results. Allowing for differences in the histological technique, and the fact that Series I animals had undergone their last molt more recently, the control animals in Series II did not differ greatly from the controls in Series I with respect to the amounts of neurosecretory materials within the corpora cardiaca. The controls of Series II had, if anything, slightly more neurosecretory material than the Series I controls which were tested closer to their time of molting. The distribution of the neurosecretory materials within the corpora cardiaca was also similar to that observed in the controls of Series I.

Animals 3, 4 and 5 of Series II had very little neurosecretory material within the corpora cardiaca, the amounts and distribution being approximately the same as found in animals 4, 5 and 6 of Series I. (Photographs A and B of Figure 1 show typical sections through the corpora cardiaca of a control (C1) and animal No. 3 from Series II.) This confirmed the previous conclusion concerning the effects of the shock treatments lasting 15 minutes. The results from this series were inadequate, however, for determining the possible rate of restoration of the neurosecretory substances within the glands. Animal No. 6 had more neurosecretory material in the corpora cardiaca than could be found in the glands of other experimental animals of this series, but the amount was still far less than in the controls. Unfortunately, not enough suitable animals were available to permit more extensive tests concerning this point, but clearly the time required for refill of

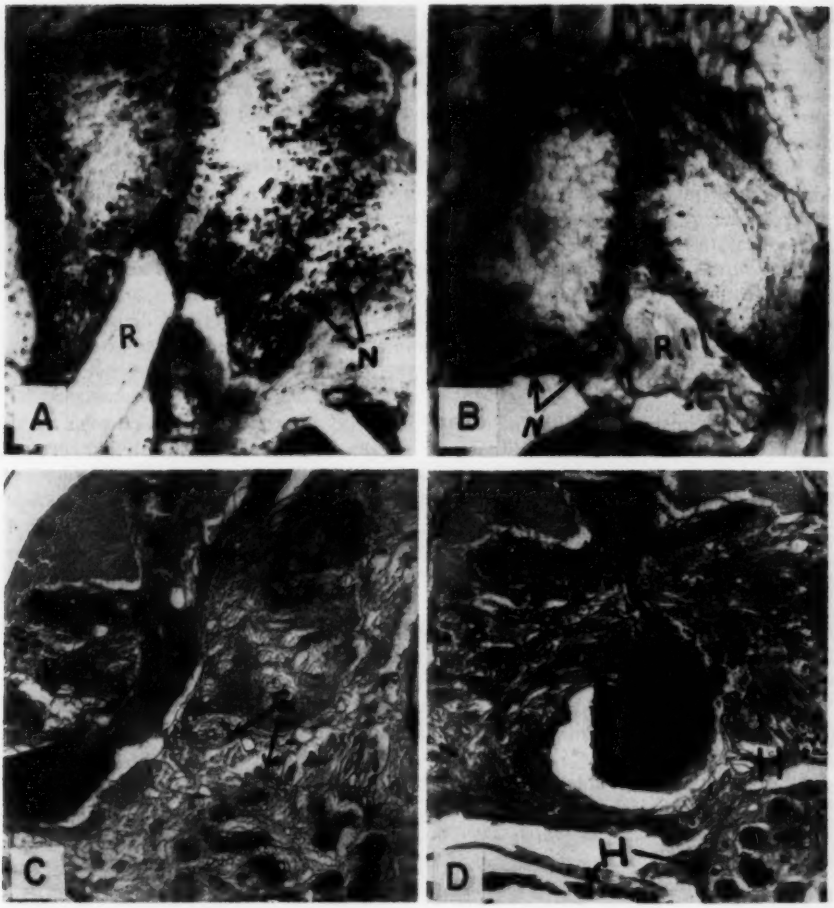

FIGURE 1. Photomicrographs of typical histological sections; all were fixed with Helly's solution and stained with aldehyde fuchsin. A—cross-section of corpora cardiaca from control animal (C1 of Series II), 144 ×; B—cross-section of corpora cardiaca from animal (No. 3 of Series II) which had received abdominal shocks for 15 minutes, 144 ×; C—cross-section of brain of an untreated control (C1 of Series III), 72 ×; D—cross-section of brain of an animal (No. 5 of Series III) which had been hyperactive for one hour, 72 ×. G—glia cells. H—hemocytes. N—neurosecretory materials. P—pars intercerebralis (containing neurosecretory cell bodies). R—recurrent nerve.

the glands to a condition resembling that of the controls must be measured in days, or possibly in weeks, rather than hours.

Series III consisted of 5 animals tested 42 days after molting to determine whether forced activity of the roaches would produce effects upon the corpora

cardiaca similar to the effects of electric shocks. Two animals were sacrificed without any experimental treatment to serve as controls; when stained with AF, their corpora cardiaca did not differ significantly from those of the controls in Series II. The experimental animals were sacrificed immediately after various periods of forced activity: one minute, 15 minutes, and one hour. After one minute of activity, no significant reduction of neurosecretory material within the corpora cardiaca was observed. There were, however, marked reductions of neurosecretory materials in the corpora cardiaca of the animals active for 15 minutes and one hour; their corpora cardiaca appeared similar histologically to those from animals given shocks for 15 minutes or longer. These results were interpreted as evidence that "stress" situations other than those caused by electric shock treatments could also induce release of neurosecretory materials from the corpora cardiaca. Certain other effects of the periods of forced activity are presented later in this section.

Series IV consisted of 6 animals tested 18 days after molting. Since Özbas and Hodgson (1958) have shown that corpus cardiacum extracts depress spontaneous nerve activity in roach nerve cords, this series was used to determine whether corpora cardiaca retain their potency for affecting spontaneous nerve activity when the glands are taken from animals which have been hyperactive. Corpora cardiaca were removed from two control animals which were not given any experimental treatment. These extracts were tested in two lots of two glands each, and it was found that they depressed spontaneous nerve activity in roach nerve cords in essentially the same manner as has been previously described by Özbas and Hodgson (1958). Extracts of corpora cardiaca from the other three animals were similarly tested after the animals had been forced to keep active for periods of 15, 60, and 120 minutes each. The glands were removed immediately after the periods of forced activity. None of the extracts of corpora cardiaca from these three experimental animals caused any significant changes in spontaneous nerve activity in the nerve cords, thus showing a clear difference from the controls and confirming the effects of the forced activity upon the neurosecretory content of the corpora cardiaca, using an entirely different method from the histological analysis previously employed.

Since the neurosecretory substances dealt with in this study come to the corpora cardiaca from cell bodies located within the protocerebrum of the roach's brain, sections were also cut through the brains of roaches in Series I through III. These sections were studied particularly with regard to any histological changes in the neurosecretory cells of the pars intercerebralis which might have resulted from the various experimental treatments. The amounts of neurosecretory materials within the cell bodies of these neurosecretory cells varied so much from cell to cell in both control and experimental groups that no significant changes could be attributed to the experimental treatments. (See photographs C and D of Figure 1 for the location of the neurosecretory cells.)

In sections of the brains of some experimental animals, there was one difference from the controls which was very striking—this difference being the presence of blood cells distributed throughout the brains of the experimental animals. This was observed in brain sections from all three experimental animals which had undergone periods of hyperactivity in Series III. At least several hundred blood cells could be seen within the brain of the roach which had been kept active for one

hour (see Fig. 1 D), and it was estimated that between one and two hundred blood cells were present in the brains of the two animals kept active for shorter periods. (Exact counts are difficult to make in these cases because the blood cells are sometimes tightly clustered and the same cells may be seen in more than one serial section.) A similar invasion of the brain by blood was also observed in sections of the brain of the one roach in Series II which was sacrificed immediately following 15 minutes of abdominal shocks. Although only 16 blood cells were counted within the brain tissue of this animal, the presence of even a few blood cells within the roach brain must be regarded as significant because no blood cells were ever observed in the brains of any of the control animals similarly stained with AF. No blood cells were seen in material from Series I (stained with CHP), and the absence of blood cells in the other experimental animals of Series II suggests that the invasion of the brain by hemocytes is a temporary one, possibly lasting even less than an hour, although this is really a separate problem which cannot be analyzed adequately from the present results. Some of the other problems raised by this movement of hemocytes will be discussed below.

Discussion

The experimental induction of the release of neurosecretory materials reported here is analogous to the results obtained in several other cases involving both arthropods and mammals. Kleinholz and Little (1949) found that asphyxia, like injection of eyestalk extracts, produced hyperglycemia in the crab *Libinia*. It was later proven conclusively that the mediation of the sinus gland within the eyestalk was essential in such cases of induced hyperglycemia in various crustaceans, and that many experimental treatments, including crowding, handling, and anesthesia of the animals, were also effective upon the sinus gland (presumably causing the gland to release stored neurosecretory substances), thereby producing hyperglycemia (Kleinholz, Havel and Reichart, 1950). The imposition of stress or injury to the animal appears to be a common feature of these experimental treatments affecting the sinus glands (Carlisle and Knowles, 1959).

An analogous case involving the rat has been reported by Rothballer (1953). Release of neurosecretory materials from the neurohypophysis of the rat was brought about by application of painful stimuli to the experimental animals, and there were indications that even handling the rats might result in loss of neurosecretory material from the neurohypophysis. Here, too, the imposition of stress would appear to be a common feature of the different treatments affecting the neurosecretory center. For similar reasons of convenience, the term "stress" is useful to indicate a common feature of the stimuli applied to roaches in the present experiments—that is, these stimuli would be expected to produce discomfort, pain, fatigue, or exhaustion, and to elicit rapid secretion of hormones from the adrenal medulla of a mammal. No identity of the mechanisms of response to stress in mammals and invertebrates is meant to be implied, however.

The exact mechanism linking the electrical stimulation or forced hyperactivity of the roaches and the release of neurosecretory materials from their corpora cardiaca is unknown. In the case of the electrical shocks, particularly in view of the magnitude of the shocks and their application directly within the head in some of the experimental animals, it might be reasonably argued that the shocks were

directly stimulating the axons or cell bodies of the neurosecretory cells supplying the corpora cardiaca. Potter and Loewenstein (1955) have demonstrated the conduction of action potentials along neurosecretory cell axons following electrical stimulation in the fish *Lophius*. Knowles, Carlisle and Dupont-Raabe (1955) used electrical stimulation to elicit the release of a chromactivating substance from a crustacean neurosecretory system *in vitro*. The assumption that arrival of nerve impulses, either normally or experimentally initiated, at the ends of the axons of neurosecretory cells would lead to release of neurosecretory substances from such cells is compatible with much contemporary thought concerning the release of neuroendocrine substances (Welsh, 1959).

In the case of hyperactivity involving no electrical stimulation, other mechanisms must be involved. There is already abundant evidence from studies on other neuroendocrine systems that the controlling factors may be quite complex and may exert their actions through more than one intermediary mechanism (Scharrer, 1959), even though transmission of nerve impulses along axons of neurosecretory cells may be the ultimate trigger mechanisms for release of neurosecretory substances from such accumulation centers as the corpus cardiacum. It is unfortunate that the operation of severing the neurosecretory axons between the brain and the corpora cardiaca prior to the periods of forced activity, which might otherwise be expected to test the importance of the innervation of the corpora cardiaca, is an operation which necessitates considerable handling and operative trauma to the animal. In itself, this procedure would probably affect the amounts of neurosecretory materials within the glands, as well as deprive them of their source of supply of neurosecretory materials.

Only very tentative hypotheses can be offered as to the role of these processes in the normal life of the animal.The nature of the experimental treatments which cause the release of neurosecretory materials from the corpora cardiaca suggests that the release of such materials may be part of the normal reactions to stress. It is already known that corpus cardiacum extracts increase the frequency of the insect heart beat (Unger, 1957); they also decrease, sometimes after an initial transient increase, the amount of spontaneous activity in nerve cords *in vitro* (Özbas and Hodgson, 1958), and probably other metabolic effects remain to be discovered. Although certain adrenalin-like effects of corpus cardiacum extracts (Cameron, 1953) further suggest an analogy with the secretion of adrenalin during the response of mammals to stress situations, it remains to be determined whether the compounds being released from the corpora cardiaca during experimental conditions such as used in the present study actually bear any chemical relationship with adrenalin. An inclusive interpretation of these diverse effects would be particularly aided at the present time by studies of the effects of stress on central nervous activity, heart function, etc., in the intact animal. The invasion of the brain by blood cells, which was unexpectedly found to follow certain experimental treatments, has not been previously reported. In staining characteristics, the invading hemocytes resemble those within blood sinuses (one of which is shown in the lower left corner of Fig. 1 D). The hemocytes do not, however, exactly resemble any of the types commonly described, but variations from species to species, and with different stains, make such precise identifications difficult even under the best of conditions (Munson, 1953). The hemocytes were not localized in any one part of the brain, and no visible damage to tissues of the brain appeared to follow the experimental

treatments; these facts would appear to rule out simple hemorrhage or phagocytic action as an explanation for the distribution of the hemocytes. There is some evidence that hemocytes store, transport, and transform nutritive materials (Munson, 1953), and such might be their functions during the experimental treatments administered in the present cases. Further studies on this problem are planned.

The authors take pleasure in thanking Dr. Berta Scharrer for her helpful discussions of this work and critical reading of the manuscript. The responsibility for any errors and for the conclusions, however, is entirely our own.

Summary

1. Neurosecretory materials within the corpora cardiaca of adult female roaches (*Blaberus craniifer*) were studied histologically, using chrome hematoxylin and phloxin or aldehyde fuchsin stains.

2. Both the neurosecretory substances which are stained dark blue with chrome hematoxylin and those stained pink by phloxine are markedly depleted in the corpora cardiaca following administration of electric shocks for periods of 15 minutes or more to the heads or abdomens of the roaches.

3. Forced hyperactivity of the roaches, when continued for periods of 15 minutes or longer, also causes a marked decrease in the same neurosecretory materials within the corpora cardiaca.

4. Following periods of forced hyperactivity of the animals, there is also a loss of the potency of extracts prepared from their corpora cardiaca, when such extracts are assayed for their ability to depress spontaneous activity in roach central nerve cords *in vitro*.

5. It is suggested that the release of neurosecretory substances from the corpora cardiaca may be a part of the roach's response to stress situations.

6. Hyperactivity of the roaches and, to a lesser extent, electric shock treatments, result in the invasion of all parts of the brain by blood cells. The significance of this phenomenon is not known.

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EXAGGERATED ELEVATION OF THE FERTILIZATION MEMBRANE OF CHAETOPTERUS EGGS, RESULTING FROM COLD-TREATMENT[1]

CATHERINE HENLEY

Marine Biological Laboratory, Woods Hole, Mass., and Department of Zoology, University of North Carolina, Chapel Hill, N. C.

There are many phenomena associated with elevation of the vitelline membrane after activation of invertebrate eggs; these have long been of interest to embryologists. Recently, Costello (1958a) has summarized much of the earlier work, and has contributed new observations concerning the behavior of the vitelline membrane and the jelly material which is secreted by the egg of *Nereis limbata* after fertilization. These studies involved the centrifuging and subsequent treatment of *Nereis* eggs with alkaline sodium chloride (pH 10.5), which had earlier been shown by Costello (1945) to be effective in producing exaggerated membrane elevation and denuding of the eggs. The method is based upon one first described by Hatt (1931) and subsequently modified by Novikoff (1939) for the eggs of *Sabellaria vulgaris*.

Costello (1958a) interpreted his results to indicate that centrifuging of the *Nereis* egg resulted in a displacement of the cortical jelly-precursor material, so that upon subsequent activation such centrifuged eggs had an asymmetrical perivitelline space which was widest at the centrifugal pole. The jelly-layer, which forms external to the membrane, was also asymmetrical and thickest at the same area. When centrifuged unfertilized *Nereis* eggs were treated with alkaline NaCl, there was a retention of the jelly beneath the vitelline membrane and formation of an asymmetrical perivitelline space.

Activation of the egg of *Chaetopterus pergamentaceus* is not accompanied by cortical changes of so obvious a nature as those reported for *Nereis* by Lillie (1911), Novikoff (1939) and Costello (1949, 1958a). There are, however, inconspicuous rhythmic contractions of the vitelline membrane of the *Chaetopterus* egg, beginning about 20 minutes after fertilization, which were described briefly by Lillie (1906) and in more detail by Pasteels (1950). It was suggested by Costello (1958a) that (page 180), "These might be occasioned by rhythmic release of small quantities of colloidal material in progressive waves sweeping over the egg surface from an origin at one pole or the other." Evidence which offers support for this hypothesis has been obtained during the course of experiments involving the treatment of *Chaetopterus* eggs with low temperature.

Brief preliminary accounts of some of this work have been reported (Costello and Henley, 1949; Henley and Costello, 1949; Henley, 1958).

[1] Aided by a grant from the National Institutes of Health, U. S. Public Health Service, RG-5328, to Dr. D. P. Costello. I am indebted to Dr. Costello for a critical reading of the manuscript, and for his assistance with the photographs.

Methods

Eggs and sperm of the polychaete annelid *Chaetopterus pergamentaceus* were obtained and handled by the methods outlined by Costello *et al.* (1957). The general plan of the experiments involved the following procedure: Two to five minutes after insemination, the experimental eggs were, for those series which involved temperature shock, transferred to six-inch fingerbowls containing pre-chilled (2–3° C.), freshly filtered, aerated sea water; the fingerbowls rested in a nest of cracked ice contained in a large fingerbowl, which in turn was kept in the refrigerator. In the series of experiments which did not involve temperature shock, the eggs (contained in a six-inch fingerbowl of freshly filtered, aerated sea water at room temperature) were gradually chilled by placing the fingerbowl in a larger dish of cracked ice which in turn was placed in the refrigerator. Usually a period of about 60 minutes was required for the temperature of 2–3° C. to be reached in those experiments which did not involve temperature shock.

Appropriate controls were kept for all series; these dishes were kept surrounded by running sea water.

At the end of the treatment period, the culture dishes in all series were removed from the refrigerator and ice-bath to the sea water table, and allowed to return gradually to room temperature, which varied from 20 to 24.5° C. during the various periods of the experiments. Egg-samples were removed and studied at frequent intervals during and following the warming-up period. No temperature shock was involved during the post-treatment period for any of the experiments.

Counts were made to determine the approximate time of 50% cleavage in experimental and control egg-populations, so that a quantitative measure could be obtained of the cleavage delay brought about as a consequence of treatment. All observations and photographs of living eggs and embryos were made without the use of coverslips; drops of culture fluid were examined in uncovered Columbia watchglasses.

For some series, fixed and stained whole-mount preparations were made of control and experimental eggs. These were prepared according to the method described by Henley and Costello (1957), being fixed in Kahle's fluid and stained in Harris' acid haematoxylin, or by the Feulgen technique. Most of the results reported here, however, are based upon observations of living embryos and larvae.

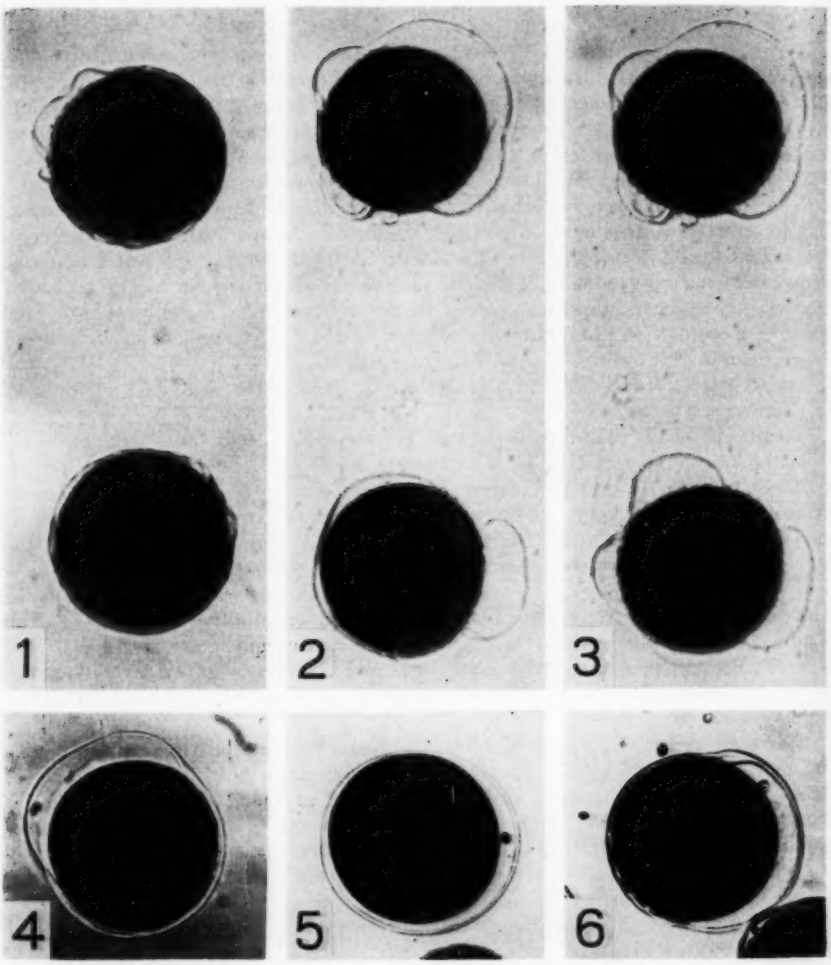
Photographs of living and fixed eggs were made using a Leica camera with Micro-Ibso attachment.

A total of over 30 experiments was performed, during the course of several summers at Woods Hole.

Results

Membrane elevation

The most striking single result of cold-treatment of *Chaetopterus* eggs is the markedly asymmetrical exaggeration of vitelline membrane elevation, which occurs during the period of gradual return of the eggs to room temperature (Figs. 1–10). This process begins rather slowly and, for the first 10 minutes or so (after moderately short periods of treatment), appears to involve no atypical changes in the egg or membrane. A relatively localized shallow crinkling of the membrane then begins at one sector (Fig. 1, lower egg); this is probably, at most, only a slight



All photomicrographs are of living, fertilized *Chaetopterus* eggs; the pictures were made with a Leica camera and Micro-Ibso attachment. Magnification of all figures after reproduction: approximately 250 ×.

FIGURE 1. Eggs cold-treated for 150 minutes with temperature shock and photographed 11 minutes after the end of treatment. In the lower egg, the first area of membrane wrinkling is visible at approximately one o'clock on the egg periphery; the elevation of the entire membrane is still at about the normal height. In the upper egg, the second area of membrane "blistering" has begun to appear, the original area of crenation still being visible at the lower pole of the egg.

FIGURE 2. Another pair of eggs, cold-treated for 150 minutes with temperature shock and photographed 21 minutes after the end of treatment. Note the enlarged "blistered" areas in the membranes of both eggs.

exaggeration of the normal membrane wrinklings which occur at the vegetal pole, beginning about 20 minutes after insemination (Lillie, 1906; Pasteels, 1950). Within a period of approximately five minutes, a second, much more pronounced, localized area of membrane elevation appears in the treated eggs (Fig. 1, upper egg). This second region is the one from which subsequent exaggerated membrane elevation proceeds. It is, from its first appearance, quite distinctive and is characterized by a more blister-like configuration of the membrane (Fig. 2). It appears to bear no constant spatial relationship to the original wrinkled vegetal pole area from which, as aforesaid, it is entirely separate.

During the course of the next few minutes, additional deep folds may appear in the membrane (Fig. 3); eventually these become contiguous and by 35 minutes after the end of treatment (approximately equivalent to 40 minutes after insemination) the membrane is smoothly and very exaggeratedly elevated from the egg surface (Fig. 10, upper egg). The course of membrane elevation during the period from 28 minutes to 33 minutes after the end of treatment is shown in Figures 7–10. These pictures show clearly that once the process of exaggerated membrane elevation begins, it proceeds rapidly (see also Figures 2 and 3, photographs taken one minute apart).

The ultimate exaggerated elevation attained may be represented by Figure 10 (top), shown 33 minutes after the end of treatment. The elevation of the membrane is quite markedly asymmetrical, the point where it is nearest the egg surface representing the sector which was first wrinkled (not that second center from which the process of elevation proceeds). Even here, however, at the point of closest approach to the egg surface, the elevation of the membrane is much wider than normal. The asymmetrical nature of this elevation is a characteristic and consistent result of cold-treatment.

Comparison of Figures 1 (lower egg) and 10 (upper egg) will reveal the magnitude of the exaggerated membrane elevation; although Figure 1 represents a treated egg, the elevation of the membrane has not yet proceeded beyond the normal stage.

It is important to note that except for the first area of shallow membrane wrinkling, the subsequent stages are probably quite separate and distinct (in degree, at least) from the normal membrane shape changes described by Pasteels (1950). His description of the initial crenated area in an egg 20 minutes after insemination

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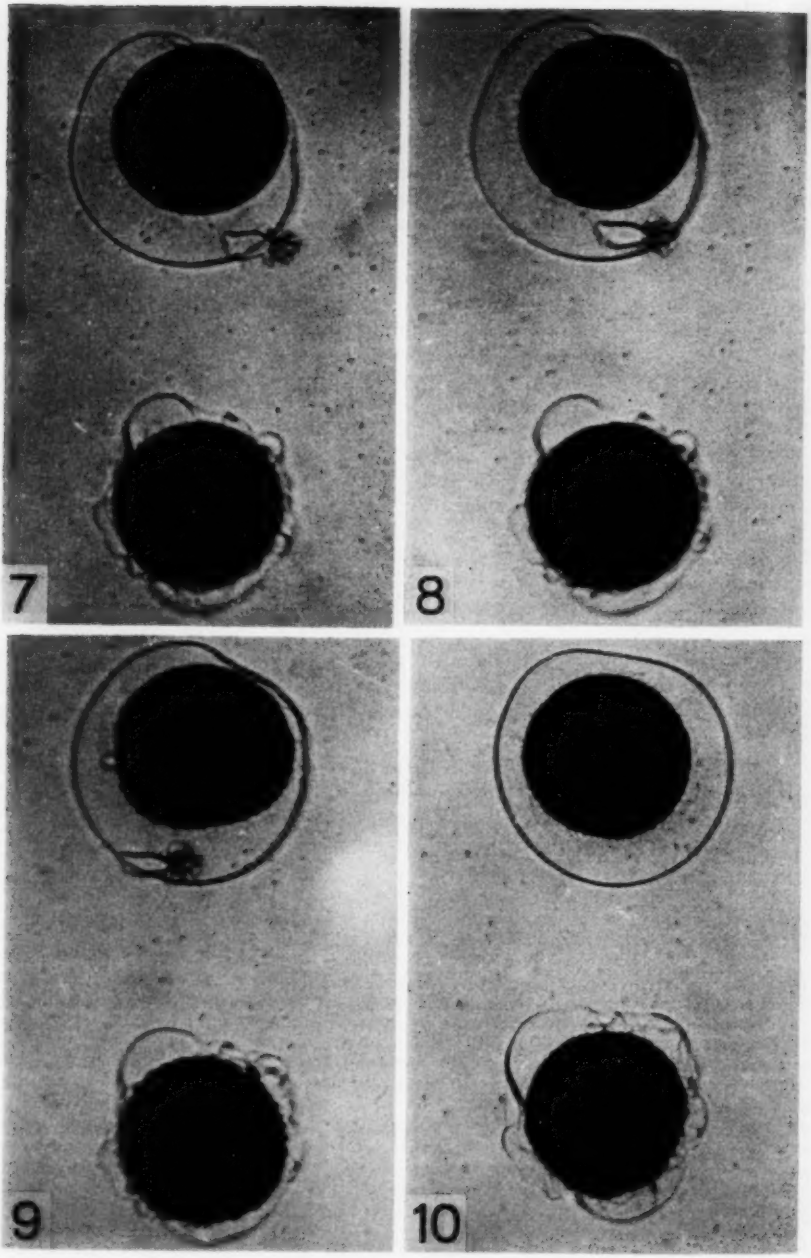
FIGURE 3. The same pair of eggs shown in Figure 2, photographed 22 minutes after the end of treatment (one minute after the photograph of Fig. 2). Note that two additional localized areas of exaggerated membrane elevation have now appeared in the lower egg.

FIGURE 4. Egg cold-treated for 6 hours without temperature shock and photographed immediately after the end of treatment. In this case, the process of exaggerated membrane elevation has begun considerably sooner after treatment than usual. The localized character of the elevation and the double nature of the membrane are apparent.

FIGURE 5. Another egg from the same treatment group as that shown in Figure 4, but photographed 50 minutes after the end of treatment. The double nature of the exaggeratedly elevated membrane is visible. The small globule between the egg surface and the membrane at the right is apparently a polar body.

FIGURE 6. A third egg from the same experimental group as those shown in Figures 4 and 5, photographed 50 minutes after the end of a 6-hour cold-treatment. The membrane has remained closely apposed to the egg surface around approximately one-half of the egg periphery but is exaggeratedly elevated around the other half. The double nature of the membrane is clear.



FIGURES 7–10.

(at an undesignated temperature) as having a "finement plissé" appearance is very accurate, and his Figure A shows that the subsequent normal wrinklings and shape changes are likewise quite shallow. We have repeatedly confirmed his findings in the study of normal, control eggs, and they are also illustrated in the photographs accompanying the paper by Harvey (1939).

There is no way of knowing with certainty whether the changes observed in our treated eggs are in any way comparable to the normal wrinklings, since the exaggerated membrane elevation is so drastic as to obscure any crenations of the type seen in untreated eggs. They are not associated with the presence of a coverslip, since all our observations of living eggs were made without the use of a coverslip. Careful study of living eggs, of fixed whole-mount preparations, and of photographs of living eggs has convinced us that the exaggeratedly elevated membrane is double in nature (Figs. 4–6).

Cleavage delay

At room temperatures of 20–25° C., the first cleavage of *Chaetopterus* eggs normally occurs from 40 to 50 minutes after insemination (Costello *et al.*, 1957). In the cold-treated eggs studied here, first cleavage for 50% of the experimental eggs was delayed from 134 to 704 minutes, the magnitude of the delay being in direct relation to the duration of treatment (Table I). As noted below in the section on the role of temperature shock, cleavage delay was much greater in those eggs subjected to temperature shock than in those which were cooled gradually (Table II), in series treated for the same length of time. From cytological study, it appears that the cold-treated eggs did not develop very far after the onset of chilling, until the treatment was ended and a gradual warming process (to room temperature) began. During the five-minute period which intervened between the time of insemination and the time when treatment was begun, the sperm penetrated the egg and, in some cases at least, the male and female pronuclei approached one another. Further development did not occur until the conclusion of the treatment. From the data reported in Table II, it appears that a period of from three to ten minutes was required for resumption of development in all cases where eggs were treated with temperature shock. For those eggs which were not abruptly chilled, there was apparently a continuation of some of the early stages of development, during the start of the cooling process, so that the magnitude of the cleavage delay was sometimes slightly less than the duration of treatment.

It is of considerable interest that even in those eggs treated for 720 minutes (12 hours), development could be resumed after cessation of the cold-treatment. As noted in Table I, the trochophore larvae developing from eggs treated for this period were very abnormal, however.

FIGURES 7–10. Successive stages of a single pair of eggs, cold-treated for 150 minutes with temperature shock. FIGURE 7: 28 minutes after the end of treatment. Note the pronounced asymmetry of exaggerated elevation in this and the succeeding photographs. FIGURE 8: 29 minutes after the end of treatment. The small particle of debris has shifted position in the interval between this photograph and that of Figure 7. FIGURE 9: 30 minutes after the end of treatment. FIGURE 10: 33 minutes after the end of treatment. The upper egg (unconfined by a coverslip) has rolled over since the photograph of Figure 9 was taken. The small piece of debris has been obscured. See text for further details.

TABLE I

The effects of cold treatment (5–12 hours) on fertilized Chaetopterus eggs

Duration of treatment	Effects on membrane elevation	Cleavage delay for 50% of exper. eggs as compared with controls	Other effects
300 minutes	Variable; some exaggeration by 15 minutes after end of treatment	301–312 minutes	Ciliary defects in trochophores. First cleavage products equal in size?
360 minutes	Ca. 90% with exaggerated membrane elevation immediately after end of treatment	320–346 minutes	Abnormal trochophores; ciliary defects; surface blebs
390 minutes	Exaggerated elevation by 45 minutes after end of treatment; eventual denuding of many eggs	429 minutes	First cleavage products equal in size?
420 minutes	94% of eggs with exaggerated elevation within 5 minutes after end of treatment	*	Abnormal, amorphous trochophores
540 minutes	90% of eggs with exaggerated elevation within 25 minutes after end of treatment	590 minutes	
720 minutes	44% of eggs with exaggerated elevation within 23 minutes after end of treatment	704 minutes	Abnormal trochophores; some very small. Ciliary defects

All treatments were begun within 2–5 minutes after insemination. The sea water medium was gradually chilled to a temperature of 2–3° C. (no temperature shock).

* The cleavages in this group of eggs were very abnormal and chaotic, and could not be timed.

Other effects of low temperature on development

The embryos and trochophores developing from the cold-treated eggs exhibited a number of characteristic abnormalities. In many of the cases where prolonged periods of treatment were used, there appeared to be a suppression of polar body formation in at least some of the eggs. This is in accord with the findings of other investigators, for other forms (notably amphibians; see the review by Fankhauser,

TABLE II

Treatments of comparable durations, with and without temperature shock

Duration of treatment	Temp. shock?	Effects on membrane elevation	Cleavage delay
180 minutes	No	None, or at most very slight	162 minutes
150 minutes	Yes	Asymmetrical exaggerated membrane elevation by 15–20 minutes after the end of treatment	152–160 minutes
120 minutes	Yes	Asymmetrical exaggerated membrane elevation by 25 minutes after the end of treatment	134 minutes
240 minutes	No	Slight exaggeration of membrane elevation in some eggs	220 minutes
185 minutes	Yes	Many eggs with exaggerated membrane elevation by 45 minutes after the end of treatment	189 minutes

1945). Less drastic exposures to low temperature apparently had no effect on polar body formation, so far as could be determined.

In a few experiments, the two blastomeres resulting from the first cleavage of treated eggs were equal or nearly equal in size. (Normally the AB and CD blastomeres resulting from the first cleavage of *Chaetopterus* and other spirally cleaving eggs are noticeably unequal.) Tyler (1930) also observed equal cleavage after cold-treatment of *Chaetopterus* eggs, but his experiments are not entirely comparable to those described in the present experiments, since he used somewhat higher temperatures, applied later in development and for considerably shorter periods of time. Tyler (1930) discusses the production of double embryos by such treatments; no evidence of double embryos, nor of the alteration of cleavage planes as described by him, was observed in the present study.

The normal egg-shape changes ("pear" and polar lobe stages) were often not clearly identifiable in the treated eggs.

The trochophore larvae which developed from cold-treated eggs almost invariably moved very sluggishly, if at all, and there were apparently severe ciliary defects. Surface blebs were often present on the trochophores. In general, the larvae bore a striking resemblance to those obtained after KCl-treatment by Lillie (1902), and after cold-treatment (10–14° C. for 14 hours) by Lillie (1906). However, we do not think that the trochophores in our cultures resulted from differentiation without cleavage, as Lillie (1906) suggested, since cytological study (see below) revealed that at least a degree of both karyokinesis and cytokinesis had occurred in all observed cases.

There was a wide variety of size among trochophores developing from eggs cold-treated for prolonged periods (12 hours). Some larvae were very small, suggesting that they might have developed from egg fragments. Such fragments, or detached cells from later cleavage stages, were observed in culture dishes of this series.

In almost all experiments there was a high mortality rate among the later embryos and trochophore larvae of the experimental groups.

Denuding of eggs

After many of the longer durations of treatment (6 hours or more), the process of exaggerated membrane elevation continued until there was a bursting of the membrane and denuding of the eggs. This denuding was very similar in course and end-results to that observed after alkaline NaCl treatment of *Nereis* and *Sabellaria* eggs. It is, however, in contrast to the process of denuding which follows alkaline NaCl treatment of *Hydroides* eggs (Costello, 1958b); there appears to be an actual dissolution of the *Hydroides* egg membrane in the alkali. (It is suggestive in this connection that Lillie, 1902, described a similar destruction of the *Chaetopterus* egg vitelline membrane in KCl and $CaCl_2$ solutions.) Subsequent development of the denuded *Chaetopterus* eggs was very abnormal under the conditions of these experiments, but no special efforts were made to cultivate such embryos further. Costello (1945) and others have shown that denuded eggs are extremely sensitive to contact with bare glass surfaces, and coating of the culture dishes with a thin layer of agar in sea water is necessary for the successful maintenance of such embryos.

After less drastic treatments, the asymmetrical exaggerated membranes retained their configuration through at least the first cleavage and denuding did not occur.

The role of temperature shock

Table II describes the results of experiments involving comparable durations of treatment time, with and without temperature shock. Even relatively short periods of cold-treatment (120 minutes) involving rapid transfer of the eggs from an ambient medium at room temperature to one pre-chilled to 2–3° C. were effective in producing a marked delay of the first cleavage, as well as the characteristic exaggerated membrane elevation. In contrast, cold-treatments of as long as 240 minutes without this temperature shock resulted in, at most, very slight effects on cleavage time and on membrane elevation. Thus, in one experiment, a treatment of 180 minutes without temperature shock resulted in a delay of 162 minutes for the first cleavage and produced only slight effects on membrane elevation. A similar treatment of 185 minutes with temperature shock resulted in a 189-minute delay of the first cleavage time for 50% of the experimental eggs (as compared with 50% of the controls); by 45 minutes after the end of treatment, there was a marked exaggeration of membrane elevation in many of the treated eggs.

Temperature shock involves the beginning of action of cold considerably sooner after insemination (five minutes) than the absence of temperature shock, where at least 60 minutes may be required to attain the treatment temperatures of 2–3° C. This suggests that the effective action of cold in producing exaggerated membrane elevation occurs within the first hour of treatment; in a gradually cooling medium, there may be opportunity for at least a semblance of the normal release of cortical material to occur, whereas this release is inhibited almost immediately if the eggs are plunged into pre-chilled sea water five minutes after insemination.

Even treatment without temperature shock, however, results in abnormal trochophores and ciliary defects, despite the fact that there may be little or no membrane exaggeration. The implication here is that the effects on membrane elevation may be very different from those affecting subsequent morphogenesis. The low temperature may thus be said to have both a direct and a delayed type of action.

Study of fixed eggs

In one typical group of eggs, cold-treated for 150 minutes with temperature shock, and fixed 15 minutes after the conclusion of the treatment (170 minutes after insemination), the normal quota of 9 chromosomes at the metaphase of the first maturation division was present and countable in most instances. In some eggs, the sperm nucleus was still visible in the interior of the egg, as a separate entity; in others, approach and fusion of the pronuclei had advanced further, and the male and female components were no longer separable on the basis of appearance. Control eggs fixed at the same time were proceeding from the eight- to the sixteen-cell stage in a normal fashion. Experimental eggs of the same series fixed 205 minutes after insemination (50 minutes after the end of treatment) were at the metaphase of the first cleavage, while control eggs were at the metaphase of the fifth cleavage. Several multipolar spindles were observed among the experimental eggs of this group.

The cortical regions of the experimental eggs fixed 50 minutes after the end of treatment presented a striking picture. There was a marked asymmetry of cortical material, apparent as a more lightly staining peripheral band with its greatest width within one relatively localized sector of the egg circumference. These eggs were fixed, whole, on coverslips and there is inherent in the method a certain minor degree of distortion of the egg shape. However, we observed no comparable asymmetry of the cortical material in control eggs of this or any other series, and therefore suggest tentatively that this may represent the area where cortical colloid material has been released in the observed asymmetrical fashion. The eggs of this group were marked by a high incidence of asymmetrical membrane elevation, which was first apparent about 30 minutes after the end of treatment, or 20 minutes before the eggs were fixed. The asymmetry of the cortical area is not visible in the experimental eggs which were fixed 15 minutes after the end of treatment; these ova, in the living condition, had not yet undergone exaggerated membrane elevation.

In addition to the multipolar spindles mentioned above, a number of other types of cytological abnormality were noted, especially in those eggs which were fixed several hours after the end of treatment. Among the abnormalities were chromosome bridges, lagging or lost chromosomes or chromosome fragments, and unequal division of chromatin material to the two poles of the division figure. All these anomalies are typical of the kinds produced in other material as a consequence of low (and high) temperature, among other agents.

There was some evidence of the production of polyploidy in larvae from cold-treated eggs; although a few of the body cells of trochophores obviously had more than the diploid number (18) of chromosomes present, such duplications of individual chromosomes or sets of chromosomes was apparently not the rule in all cells of a given larva. The chromosomes of *Chaetopterus* are small and tend to be crowded on the spindle, so that counting them is difficult, even in early cleavage stages; to do so is almost impossible in the minute cells of later cleavage stages and trochophores. The observed anomalies of chromatin distribution may have arisen as a result of the polar body suppression mentioned above.

Cytological preparations of advanced cleavage stages of the treated eggs indicate that there was often some suppression of cytokinesis (although karyokinesis had proceeded in a variable fashion). In all cases studied, however, at least some degree of cytoplasmic division had occurred, and there was no evidence of differentiation without cleavage. As would be expected, there was a wide variation in cell size, in the treated embryos and larvae.

Discussion

The possible mechanism of action of low temperature

The characteristic asymmetry and exaggeration of membrane elevation in the cold-treated *Chaetopterus* eggs suggest that cold-treatment may interfere with the gradual and rhythmic release of some substance from the egg surface after insemination, so that a sudden localized release of such material occurs at the cessation of treatment. There may also be a change in the permeability of the vitelline membrane, so that the colloidal material is retained within the perivitelline space, bringing about the observed exaggerated membrane elevation. The asymmetry of this

exaggerated elevation could be a consequence of the accumulation of colloidal substance within one relatively localized sector of the egg, or of changes in the egg cortex resulting in release of such a substance in a considerably smaller segment of the egg periphery than is normal. The *Chaetopterus* egg secretes no external jelly after activation, and the substance whose abrupt release brings about membrane elevation in the treated eggs is therefore postulated to be of some other nature. Thus, the situation for the *Chaetopterus* egg differs, in detail at least, from that described for the *Nereis* egg by Costello (1958a).

Some additional evidence for this idea is afforded by observations on the action of gum arabic solutions (in sea water) applied to eggs with exaggerated membrane elevation. In such cases, the membranes promptly collapsed back against the surfaces of the eggs.

Pasteels (1950) has also suggested that there may be a rhythmic and localized release of some sort of material from the cortex of the *Chaetopterus* egg, during the post-fertilization period. His experiments involving treatment of eggs of this form with KCl appear to support such an idea if one assumes that the KCl, like cold, blocks the process of release during the course of the treatment.

Pasteels was able to demonstrate a direct correlation in fixed KCl-treated eggs between areas of abnormal membrane wrinkling, and cortical and membrane alterations of structure and staining capacity. This correlation appears to be comparable to that observed in the present study of fixed and stained cold-treated eggs.

Exaggerated membrane elevation as a consequence of other experimental procedures

Lillie (1902) mentioned briefly the occurrence of exaggerated membrane elevation and dissolution in KCl- and $CaCl_2$-treated fertilized and unfertilized *Chaetopterus eggs*. A more thorough study of the same problem was undertaken by Pasteels (1950), who treated unfertilized *Chaetopterus* eggs with KCl by the method of Lillie. Pasteels illustrates a process of asymmetrical membrane elevation which, in some respects, is reminiscent of that reported in the present study. His Figure B shows that by 40 minutes after the end of KCl-treatment (95% sea water, 5% 2.5 *M* KCl, for one hour), there are the beginnings of an asymmetrical membrane elevation which, at this stage, is similar to that observed after cold-treatment. The subsequent course of events after KCl-treatment, however, is quite different; by 102 minutes after the end of this treatment, there is a very asymmetrical, deeply rugose exaggeration of membrane elevation. Apparently this stage is not succeeded by a smooth state of exaggerated membrane elevation.

Redfield and Bright (1921) reported exaggerated elevation of the *Nereis* egg membrane after various types of irradiation. Following beta or gamma radiation, the elevation was symmetrical; after alpha or ultraviolet treatment, it was asymmetrical. Redfield and Bright state that they did not obtain an increase in the volume of the perivitelline space after the irradiation of eggs such as *Cumingia, Asterias, Arbacia* and *Chaetopterus*, which do not normally produce jelly as a part of the fertilization reaction. However, Moser (1939), Spikes (1944) and Rustad (1959) reported the asymmetrical elevation of the sea urchin egg fertilization membrane after ultraviolet irradiation of one side of the eggs. It is of interest that x-irradiated unfertilized *Chaetopterus* eggs (treated with 20,000 and 40,000 r

and then inseminated) showed no exaggeration of membrane elevation (Henley, 1958). Furthermore, whole-mount preparations of such x-irradiated eggs, fixed at the metaphase of the first cleavage, do not reveal the asymmetry of cortical material reported above for cold-treated eggs. Although this observation is by no means conclusive evidence, it does suggest that the appearance of such cold-treated ova is associated with the postulated abnormal retention and delayed release of cortical material.

As noted in the introduction, alkaline sodium chloride treatment (pH 10.5) results in a drastic exaggeration of membrane elevation in the *Nereis* egg (Costello, 1945; Lovelace, 1949); this exaggeration, unlike that reported here, is symmetrical in nature, except for the circumscribed area of sperm entrance. Alkaline NaCl-treatment of *Chaetopterus* eggs is followed by an extremely rapid elevation of the membrane, which results in denuding of the eggs in less than five minutes. The exact process involved will be described in a later communication; suffice it to say here that the membrane elevation appears to be only superficially comparable to that produced as a result of cold-treatment.

Moser (1939) illustrates asymmetrical (although not especially exaggerated) membrane elevation in saponin-treated unfertilized *Arbacia* eggs.

The chronological relationships of events in cold-treated vs. control eggs

It is interesting to compare the chronological relationships of events in the cold-treated eggs and in the normal, untreated eggs. Pasteels (1950) has described the following series of membrane shape changes and wrinklings in fertilized *Chaetopterus* eggs; he did not specify the room temperature but, from the times noted for several "landmark" events, this may be assumed to have been about 17–20° C., somewhat lower than those prevailing during our experiments.

20 minutes after insemination: There is a vegetal-to-animal pole wave of shallow membrane wrinklings.

23 minutes after insemination: The first polar body is given off; from now until after the second polar body appears, the membrane remains smooth.

30 minutes after insemination: The second polar body is given off.

32 minutes after insemination: A second vegetal-to-animal pole wave of membrane wrinkling occurs.

38 minutes after insemination: "Pear-shaped" stage; there is now an animal-to-vegetal pole wave of wrinkling in the membrane, reversing the first two waves.

42 minutes after insemination: Polar lobe stage; wrinkling of the membrane now occurs in a wave from *each* pole, more or less simultaneously, leaving an equatorial band around the egg free of wrinkles.

66 minutes after insemination: First cleavage begins; the wrinklings of the membrane are accentuated.

Our observations showed that for all experiments, both with and without temperature shock, the greatest incidence of exaggerated membrane elevation occurred immediately before the first cleavage (at a stage corresponding to the polar lobe although, as noted above, the normal shape changes of the egg cytoplasm were often not recognizable in the treated eggs). This is a time, of course, when a number of

important events are occurring within the egg, in preparation for the first cleavage. It does not seem unreasonable to suppose that the double wrinkling (animal-to-vegetal and vegetal-to-animal) described by Pasteels as occurring shortly before the first cleavage, or some feature of this phenomenon, may be exaggerated to result in the reported events.

Summary

1. Fertilized eggs of the polychaete annelid, *Chaetopterus pergamentaceus*, were cold-treated for various periods of time, ranging from 150 to 720 minutes, beginning immediately after insemination. Two general methods were employed; in one, the eggs were plunged into pre-chilled, filtered aerated sea water (2–3° C.); these experiments are referred to as involving temperature shock. In the other type, the eggs were gradually chilled to approximately the above temperature. At the end of the treatment period all eggs were allowed to return gradually to room temperature.

2. When eggs were cold-treated, with or without temperature shock, there was a pronounced asymmetrical exaggerated elevation of the vitelline membrane, which reached its greatest incidence about 40 minutes after the end of treatment, or shortly before the first cleavage. This exaggerated elevation continued in some cases after prolonged cold-treatment, so that the eggs were eventually denuded.

3. Most of the cold-treatments used were followed by delays in the first cleavage time for 50% of the experimental eggs as compared with 50% of the control population.

4. In all cases where cold-treatment was initiated with temperature shock, the effects on membrane elevation and cleavage time were much more pronounced than when the eggs were chilled gradually.

5. A number of characteristic morphological and cytological abnormalities were noted in embryos developing from the treated eggs; these included severe ciliary defects, fragmentation of the embryos, lagging or lost chromosomes or chromosome fragments, duplication of chromosome sets and/or individual chromosomes, suppression of polar bodies, and multipolar spindles.

6. It is suggested that the findings reported here afford evidence supporting Costello's (1958a) hypothesis that the rhythmic wrinkling and shape changes reported for the normal *Chaetopterus* egg by Pasteels may be due to the gradual release of some colloidal material from the surface of the egg. This release is considered to be blocked in some manner by the low temperature, and when the treatment is terminated, the release proceeds in a drastic, non-rhythmic manner; it then appears to be coupled with a change in the permeability of the vitelline membrane, so that the colloidal material is retained between the egg surface and the membrane. This brings about the exaggerated membrane elevation observed.

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HISTOCHEMICAL STUDIES ON THE NATURE AND FORMATION OF EGG CAPSULES OF THE SHARK CHILOSCYLLIUM GRISEUM [1]

G. KRISHNAN

Department of Zoology, University of Madras, India

Pryor (1940) demonstrated that the ootheca of *Blatta* is composed of a tanned protein similar to that of insect cuticle. Since then a number of authors reported the occurrence of tanned proteins in the cysts of nematodes, egg shells of helminths, setae of earthworm and byssus of *Mytilus edulis* (Ellenby, 1946; Stephenson, 1947; Dennell, 1949; Brown, 1952; Smyth, 1954). In this connection it is of interest to recall the observation of Brown (1950a) that the egg capsules of selachians also give evidence of phenolic tanning although it was believed by the earlier workers to be formed of a material similar to keratin. In the light of these observations it was thought that a study of the nature and composition of the egg capsules of shark would be of interest in itself and for comparison with the egg shells of helminths and ootheca of *Blatta* which have been shown to possess a protein constitution resembling in essential respects that of tanned cuticles of insects.

The materials used in the following study comprise nidamental glands and egg capsules collected from gravid females of *Chiloscyllium griseum*. The staining and histochemical reagents used are mentioned in relevant context. For localization of the oxidase of the egg capsule, the "catechol" technique (Smyth, 1954) was applied. The microchemical and chromatographic procedures employed for the study of the protein of the egg capsule are described in the text.

Egg Capsule—Structure

The egg capsules of sharks and rays have been described by a number of workers (Beard, 1890; Widakowich, 1906; Clark, 1922; Hobson, 1930; Nalini, 1940). The shell material is said to be secreted by the cells of the nidamental gland and the egg capsule is formed in the caudal part of the oviduct. The sequence of events in the formation of the egg case is not known for certain. There is some evidence to suggest that a major part of the egg case is formed before the arrival of the fertilized egg. The egg case with the enclosed egg is later ejected into the sea.

The egg capsules of sharks are more or less rectangular in shape with the corners prolonged into anterior and posterior pairs of horns, but considerable variation exists in shape and size in the different species. There is little precise information regarding the nature of the material composing the egg capsule. The earlier workers referred to it as chitinous but the term as used by them carried no chemical significance. On the other hand Hussakof and Welker (1908) who re-

[1] This work was carried out in the Department of Zoology, University of Madras. I am thankful to Dr. E. R. B. Shanmugasundaram of the Department of Biochemistry for help and advice in regard to the chemical analysis reported in this paper.

ported on the chemical nature of the egg case of two species of sharks, suggested that it may be similar to keratin. However, the previous workers are agreed that in all selachians the structure and composition of the egg capsule are identical. Widakowich (1906) noted that in *Scyllium* the egg capsule is formed of a large number of "Platten" which adhere to each other loosely at first and later, especially after contact with sea water, much more closely so that the entire capsule hardens. It has also been observed that the capsule when first formed within the oviduct is white and soft and gradually hardens, undergoing a change in color to brown and later to deep reddish brown. In *Chiloscyllium griseum* the egg capsules taken from the oviducts show a range of coloration varying from very light brown to deep reddish brown. The ridge-like thickening bordering the capsule is more deeply colored than the rest of it.

Frozen sections of the capsule wall, which is lightly colored, show an outermost narrow yellowish layer containing dark granular inclusions. Internal to it is a

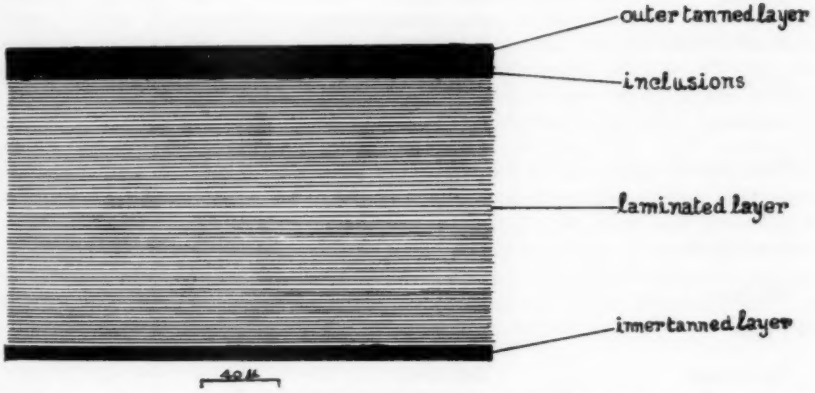

FIGURE 1. Section through the wall of a light colored egg capsule stained with Mallory.

broad region more or less uncolored and characterized by horizontal laminations. On the inner border of the laminated region is a very narrow strip which is yellow colored and apparently homogeneous, similar to the outermost layer (Fig. 1). In the laminated region a central part may be distinguished by its darker shade. The color disappears after treatment with ethylene chlorhydrin and the entire section is marked by a diminution of the dark color. This reaction may suggest the presence of melanin-like substances in the wall of the capsule (Lea, 1945). Further support to the above suggestion is obtained by the results of tests performed on the sections of the capsule wall with hydrogen peroxide and potassium permanganate, both of which produce a bleaching effect (Pearse, 1954).

In paraffin sections stained with Mallory, the outer- and innermost layers, which are yellowish in unstained preparations, are colored red with acid fuchsin while the laminated region is stained blue. Similar results were obtained with Masson's trichrome stain; the regions coloring red with Mallory are stained with xylidene ponceau and the laminated region is colored green. With Heidenhain's haema-

toxylin the outer and inner layers are dark blue while the rest of the thickness of the wall comprising the laminated zone is very lightly or not at all stained. In the regions of the capsule which are reddish brown, the staining reactions are different from those reported above. The central wide laminated region, instead of staining uniformly green or blue with Masson's and Mallory stains, shows red patches filling up the greater extent of this region. Such a change in staining reaction may suggest that the substance originally present has undergone a transformation so as to resemble that present in the outermost layer.

TABLE I

Responses of egg capsule wall of Chiloscyllium griseum to chemical tests

Test	Egg capsule wall	Outer layer	Middle laminated zone	Inner layer
Millon's	light brown	+	−	+
	deep reddish brown	++	++	++
Xanthoproteic	light brown	+	−	+
	deep reddish brown	++	++	++
Biuret	light brown	−	+	−
	deep reddish brown	−	−	−
Argentaffin	light brown	++	+	++
	deep reddish brown	++	++	++
Ferric chloride	light brown	+	++	+
	deep reddish brown	+	+	−
Ammonium molybdate	light brown	+	+	+
	deep reddish brown	+	+	+
Sudan Black B	light brown	−	−	−
	deep reddish brown	−	−	−
Chitosan	light brown	−	−	−
	deep reddish brown	−	−	−
Pepsin	light brown	−	+	−
	deep reddish brown	−	−	−
Lead acetate	light brown	−	−	−
	deep reddish brown	−	−	−
Sodium nitroprusside	light brown	−	−	−
	deep reddish brown	−	−	−
Dilute mineral acids	light brown	−	+	−
	deep reddish brown	−	−	−
Boiling H_2O	light brown	−	+	−
	deep reddish brown	−	−	−

+ = positive.
− = no apparent effect.

CHEMICAL COMPOSITION

To test the validity of the above assumption, the nature of the principal chemical components of the capsule walls yielding the staining reaction reported above was investigated. It is known from previous work that the major constituent of the shell substance is protein. In the following study a qualitative estimate of the protein constituents was made by color tests and the results are summarized in Table I. It is seen that the entire thickness of the capsule wall is positive to tests for protein. But the light colored capsules differ in some respects from those

which are deep reddish brown. It has been pointed out that the capsule when first formed is white and later turns to light brown which gradually deepens to dark reddish brown, the changes in coloration representing different phases in the growth of the capsule. In the earlier phases of growth when the capsule is very light brown, the outer and innermost layers give positive Millon's and xanthoproteic reactions, while the central laminated region is negative to these tests, but positive to biuret test. In the more fully formed capsule, which is deep reddish brown, the central laminated zone also is positive to Millon's and xanthoproteic tests. These changes in the reaction to protein color tests coincide with those in staining reactions with Mallory. Presumably the fuchsinophil substance staining red with Mallory is the same as that giving a Millon-positive reaction.

A positive reaction to Millon's test has been interpreted as indicative of a protein containing the hydroxyl-phenyl group in the molecule, and since tyrosine is the only amino acid containing it, it may be inferred that in the egg capsule Millon-positive sites may contain a protein rich in tyrosine (Pearse, 1954). The coincidence of the Millon-positive regions with those giving positive xanthoproteic reactions supports the above suggestion since the latter test is said to indicate protein containing tyrosine, tryptophane and phenylalanine (Pearse, 1954). On the basis of the positive results obtained with the above two tests, Blower (1951) suggested that the presumptive exocuticle of the myriopods studied by him contains a protein, rich in phenolic groups and which are involved in the tanning of the exocuticle. Similar results have been reported by Krishnan (1956) in the cuticle of *Scolopendra*. In the light of the above observations, a reasonable interpretation of the results of tests on the egg capsule is that when first formed it is constituted of a simple protein positive to biuret test and soon after, the outer and inner layers are modified by the presence of a protein rich in phenolic groups which appear to spread throughout the thickness of the wall. These changes are correlated with a deepening of the color of the capsule and also an increased chemical resistance.

Evidence for Quinone-Tanning

The features noted above recall strongly the characteristic change undergone by the insect cuticle during hardening by tanning (Dennell and Malek, 1955) and appear significant as indicative of a similar tanning process in the egg capsules. Brown (1950b) pointed out that if a structural protein dissolves only in sodium hypochlorite solution and is itself secreted by tissues containing polyphenols, there is circumstantial evidence for quinone tanning. It will be shown in the sequel that both the tests are positive with the egg case material. When small pieces of dark brown capsule wall were treated for varying periods with a dilute aqueous solution of sodium hypochlorite, the color was readily lost and on continued treatment the egg capsule wall was dissolved. Further evidence of tanning is indicated by the presence of a phenol oxidase which is known to be an essential participant of the tanning process in insect and crustacean cuticles (Bagvat and Richter, 1938; Dennell, 1947a, 1947b; Krishnan, 1951). In recent studies on the formation of helminth egg shell, which involves quinone tanning, the oxidase concerned has been demonstrated by the red color produced on incubation with a dilute solution of catechol (Smyth, 1954). The mechanism of the above reaction is due to the

oxidation of catechol to quinone and its condensation with the protein so as to produce a tanning effect. This technique was applied to the egg capsules of shark with positive results. Light colored capsule walls when subjected to the catechol treatment changed to a dark red color resulting from the tanning of the protein. Such a color change is less intense with dark colored capsules. That the change in color after catechol treatment is really due to the tanning of the protein may be inferred from the observation that the color is lost on addition of a dilute solution of sodium hypochlorite. Further, sections of material deeply colored by catechol treatment when stained with Mallory show correlated change in staining reaction, the central laminated region being fuchsinophil, so as to simulate the condition of a more full grown and normally reddish brown capsule. It would appear that by treatment with catechol the protein of the central layer is artificially tanned. The color change noted above was inhibited by cyanide in a concentration of 0.001 *M*, suggesting the enzymatic nature of the process and the role of an oxidase in bringing about the tanning effect.

The above observations suggest that the egg capsule after its formation undergoes a process of hardening by phenolic tanning before being ejected into the sea, and this would account for the change of its coloration from light brown to deep reddish brown. The principal participants in the process appear to be a protein probably rich in tyrosine and a phenol oxidase. The results of histochemical tests on the egg capsule material (Table I) indicate an absence of lipids which are usually associated with tanning in the cuticles of insects and other arthropods. Further, although diphenols are indicated in the capsule walls, as may be inferred from positive ferric chloride tests, in the absence of a correlation between their accumulation and the tanning of the protein, their mere presence may not indicate that they are involved in tanning. Their persistence in the outermost layer, where tanning is more intense than in the rest of the thickness of the wall, may militate against the view that they are the tanning phenols. It is suggested that they may be related to the formation of melanin occurring in the capsule walls, for diphenols, indicated by ferric chloride, accumulate in the laminated zone early in the growth of the capsule and their partial disappearance is followed by the occurrence of melanin. This feature, together with the presence of a phenol oxidase in the capsule wall, may suggest the oxidation of phenols to melanin. Since the latter appears even before the onset of tanning in this region it is suggested that the free diphenols may not be directly involved in the tanning process.

Histochemistry of the Nidamental Gland

With a view to investigate further the nature of the tanning process, a study of the mode of formation of the egg shell material was made. It is known from previous work that the materials forming the egg case are secreted by the nidamental gland. The gland is a dilatation of the oviduct at the junction of the caudal and cranial parts comprising a glandular body formed of tubules in a more or less parallel series. The histology of the gland in *Chiloscyllium* shows close agreement with that of *Scyllium canicula* and *Scyllium catalus* (Nalini, 1940). In the anterior part of the gland, distinguished as the albumin gland, the secretory tubules are formed of both glandular and ciliated cells. The secretions are in the form of transparent cytoplasmic granules which appear to be extruded by rupture of the cell

walls into the lumen of the gland tube. The succeeding section of the gland, which is distinguished as the shell-secreting zone, is formed of cells whose cytoplasm is packed with granules during the period when egg capsules are being formed. The shell substance appears to be derived from these secretions and in the light of the foregoing observations on the egg capsules, one would expect to find in these cells the constituents of the tanning system. Accordingly histochemical tests for phenols and proteins were applied. The argentaffin, ammonium molybdate and sodium iodate tests for phenols were positive in the cytoplasm of the cells of the shell-secreting zone. Identical regions of the cells were also positive to biuret tests for protein. Malachite green, which is known to show a specificity for proteins involved in tanning of egg shells of helminths (Johri and Smyth, 1956), gave positive reaction in the cytoplasm, the granules taking a vivid green color. The green coloration is said to be due to the dye becoming bonded to the protein. Since the cytoplasmic granules in the cells of this region react positively to both the tests for phenols and proteins, it is suggestive that the substance reacting may be a phenolic protein, similar to that reported to occur in the vitelline gland cells of helminths (Smyth, 1954). Frozen sections of this region of the nidamental gland when treated with a dilute solution of catechol develop readily a brown coloration in the cytoplasm of the cells. This reaction may suggest evidence of the occurrence of a protein undergoing tanning and an oxidase in close proximity to it, responsible for the oxidation of phenols involved in tanning. It appears probable that the oxidase and the substrate are both located in the cytoplasm of these cells.

Nature of Egg Capsule Protein

The foregoing observations indicate that the principal constituent of the egg capsule is a protein secreted by the cells of the nidamental gland, along with an oxidase capable of oxidizing catechol to quinone. In the egg capsules two apparently distinct protein constituents seem to occur, one forming the basal matrix which persists for some time in the central laminated region and the other being a tanned protein which is distinguished from the former by the chemical and staining reactions. In these respects they present very strong resemblance to the basal protein and that impregnating the regions destined to be tanned in the cuticle of insects like *Periplaneta* (Dennell and Malek, 1955). Here the basal protein of the procuticle stains blue with Mallory, is negative to Millon and xanthoproteic tests and lacks chemical resistance, while that impregnating the presumptive exocuticle stains red with Mallory, is positive to Millon and xanthoproteic tests and possesses considerable chemical stability. That the above characteristics of the protein of the presumptive exocuticle may be due to some sort of aromatic bonding is suggested by the observation of Kennaugh (see Dennell, 1958) that the staining properties can be reversed by treatment with Diaphanol which is known to break up the aromatic bonds by oxidation. The change in staining reaction with Mallory from red to blue, reported by the above author, may indicate a restoration of the protein component to its original state, as is found in the untanned endocuticle. In the egg capsule of the shark it is suggestive that the tanned protein is derived from the basal protein such as is found in the laminated region in the earlier stages of capsule formation. If it is so, it may be possible to restore the tanned protein to the original state by breaking up the aromatic bonds as has been done in the insect

cuticle referred to above. This was carried out by adopting the method used by Dennell (1958) who following Trim (1941), separated the tanning phenols of the puparia of *Calliphora* using alkaline stannite solution for breaking up the quinone bonds. Accordingly, small pieces of egg capsule material were left in a mixture of 2% sodium hydroxide and stannous chloride at 37° C. for nearly a week, by which time the protein was solubilized. The protein fraction was separated and tested. Unlike the tanned protein it was negative to Millon's test and was easily digested by pepsin-hydrochloric acid and showed a marked swelling in boiling water.

These observations, in addition to suggesting that the tanned protein of the egg capsule may be a derivative of the basal protein, also give some indication of the nature of the protein. The reaction to pepsin and swelling in boiling water are suggestive, especially in the light of the observation of Astbury (1945) that the entire egg capsule of shark yields an x-ray diffraction pattern similar to that of a collagenous protein.

With a view to test further the suggestion made above, a microchemical analysis of the capsule protein was made using a modification of the method of Spencer, Morgulis and Wilder (1937), who applied the above method for a determination of collagen content of the muscles of rabbit. The capsule walls were cut into small bits and cleaned by scraping with a blunt scalpel to remove all adhering tissue. A sample weighing 0.1 gm. was homogenized with an equal quantity of distilled water in a Potter-Elvehjm homogenizer, and the material was placed in a water bath at 100° C. for about 15 minutes along with 10 times its weight of water. This was later stored in a refrigerator, and next day, it was autoclaved for 3 hours at 20 pounds pressure so as to convert collagen, if any, into gelatin. The material was then centrifuged at 4,000 rev./min. for one hour and the supernatant fluid drawn off. An aliquot of this fluid was treated with 3% tannic acid when a copious precipitate was obtained. The above evidence in support of the view that a collagenous type of protein occurs in the egg capsule was checked by a chromatographic analysis of the precipitate. The material was treated with ten times its weight of 6 N HCl in a sealed tube and hydrolysed at 105° C. for 24 hours. The hydrolysate was dried in a vacuum desiccator containing potassium hydroxide and this was used for analysis by partition chromatography, following the capillary ascent method of Williams and Kirby (1948). The hydrolysate was dissolved in a small quantity of distilled water and a 20-μl sample was used for spotting on the filter paper and the chromatogram run with butanol-acetic acid-water as the solvent. Simultaneously a number of chromatograms were run under identical conditions using pure amino acids for purposes of comparison. A solution of 0.1% ninhydrin in butanol was used for spraying. Qualitative analysis of the chromatogram thus obtained shows in general an agreement in amino acid make-up with that of mammalian connective tissue (Bowes and Kenten, 1949), suggesting that the protein in question may be allied to collagen. Further, the pattern of spots was more or less identical with that of a sample of pure gelatin hydrolysed and otherwise treated in the same way as the test material. Most of the amino acids found in the chromatogram of the egg case material correspond to those found in the gelatin.

However, it is seen that the egg case material differs in the absence of hydroxylysine, leucine and valine as well as in the presence of tryptophane. The absence of hydroxylysine may suggest a relationship to elastin, but the occurrence of tryptophane is unusual for a collagenous type of protein. It is possible that its

presence may be due to a contaminant. However, it must be mentioned that the amino acid composition of collagen derived from different sources may vary markedly. The collagen of fish skin is known to differ from mammalian collagen in having a low hydroxyproline content while serine, threonine and methionine are in greater amounts (Gustavson, 1956). Such quantitative variations occur not only in those amino acids considered to be characteristic of collagen but also in some of the non-typical residues like tyrosine. A quantitative amino acid analysis is therefore necessary for making a valid comparison. However, the present study is essentially from a biological viewpoint and such evidence as has been obtained is enough to indicate the nature of the material composing the egg capsule. The presence of non-polar amino acids like glycine and alanine, the prominence of proline and hydroxyproline and the comparative rarity of aromatic residues are features of the egg capsule protein, which together are suggestive that it may be allied to the collagen group (Gustavson, 1956).

Discussion

The foregoing observations suggest that the egg capsules of *Chiloscyllium* undergo a tanning process resulting in acquisition of mechanical rigidity and chemical resistance. The process is comparable to that occurring during the formation of ootheca of *Blatta* (Pryor, 1940) but certain differences are significant. Unlike in the insect, here the substrate involved in tanning is a protein without a lipid component. No free diphenol appears to participate in the process. The resultant tanned product is also different from the tough amber colored sclerotin, being only yellowish, and retains a reactivity to stains. The protein constitution of the egg capsule appears to be such that it cannot yield sclerotin after tanning, for it has been observed in arthropod cuticles that unless the protein precursor of tanning is impregnated with a lipid constituent, sclerotin may not be the resultant product. Sclerotin itself has been considered as a lipoprotein subsequently tanned. It is clear that the tanned protein of the egg case is not sclerotin but recalls in its chemical and staining reactions the tyrosine-rich protein precursor of sclerotin, found in the presumptive exocuticle of an inset like *Periplaneta* (Dennell and Malek, 1955) or the so-called pro-sclerotin described by Blower (1951) in the myriopods studied by him. In the above instances the protein in question shows considerable chemical stability even before forming a complex with the lipid participant of tanning and stains red with Mallory, unlike the protein confined to those regions which do not undergo tanning. The chemical stability of the protein has been attributed to the occurrence even at this stage of some kind of aromatic tanning which is distinct from the subsequent tanning of the lipoprotein complex by free diphenols resulting in sclerotin (Dennell, 1958). Such a tanning has been distinguished by the above author as "primary tanning" in contrast to the "secondary tanning" which results in sclerotin. In the absence of the participation of free diphenols "primary tanning" would be presumably by oxidation of tyrosine side-chains of the protein. The tanned protein of the shark egg capsule recalls strongly the product of "primary tanning" in its chemical nature, staining characteristics, possession of resistant qualities and retention of a "tannable" condition having still free amino groups. It seems probable from the observations reported in the present study that the mode of tanning of the egg capsule protein may involve a process of auto-quinone tanning similar to that suggested to occur in the egg shells of helminths (Smyth, 1954).

Summary

1. The egg capsules of *Chiloscyllium griseum*, when first formed in the oviducts, are soft and white and gradually turn light brown to deep reddish brown before being ejected into the sea.

2. Light brown capsule walls show in section an outer and an inner narrow layer apparently homogeneous and yellowish in color while a wide central region is laminated and uncolored. This layer stains blue with Mallory, indicates the presence of a simple protein positive to biuret test and lacks chemical resistance. The outer and inner layers stain red with Mallory and contain a protein which is positive to Millon and xanthoproteic tests indicative of phenolic groups. In deeply colored walls the central laminated layer shows staining and histochemical reactions similar to those of the outer layer.

3. Evidence has been presented indicating that the above changes may be due to the tanning of a basal protein involving a phenol oxidase resident in the capsule wall.

4. The constituents of the tanning system are derived from the secretions of the cells of the nidamental gland. The tanning of the egg capsule protein does not appear to involve free diphenols so that some form of auto-quinone tanning seems to occur.

5. The tanned protein of the egg capsule is unlike the sclerotin of the insect cuticle, but recalls in its staining and histochemical reactions the protein precursor of tanning impregnating the presumptive exocuticle of insects like *Periplaneta*.

6. The nature of the egg capsule protein has been investigated using microchemical and chromatographic methods. From the results obtained it is suggested that it is allied to the collagen group of proteins.

7. The results are discussed.

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THE SIZE AND SHAPE OF METAMORPHOSING LARVAE OF VENUS (MERCENARIA) MERCENARIA GROWN AT DIFFERENT TEMPERATURES

VICTOR L. LOOSANOFF

U. S. Fish and Wildlife Service, Bureau of Commercial Fisheries, Biological Laboratory, Milford, Connecticut

Among the characters to be employed for identification of lamellibranch larvae, their size at metamorphosis has been suggested by some workers (Lebour, 1938). Other students have maintained, however, that since there may be correlations between size and environmental factors, the size at metamorphosis cannot be used as a criterion for recognition of larvae. Sullivan (1948) thought that this was confirmed for mature larvae of *Mya arenaria* in Canadian waters, which she observed to metamorphose in Malpeque Bay upon reaching a length of 250 microns, as compared with a length of 415 microns reported by Stafford (1912) for larvae of the same clam in the St. Andrews region.

According to Sullivan the difference in size at setting was probably the result of the difference in the summer water temperature at these two places because larvae developing at the lower temperature (St. Andrews) needed to reach a larger size before metamorphosing. This opinion is, indirectly, in agreement with that of Erdmann (1934), who thought that the size of larvae of the European oyster, *Ostrea edulis*, at the time of swarming was regulated by the temperature at which the larvae developed.

So far, no one has offered a definite explanation as to why lamellibranch larvae should reach a larger size if grown in colder water. It seems, however, that this opinion was formed because it is widely believed that, "In many instances, and perhaps as a general rule, the size that an animal attains is greater when it is reared at low temperature" (Coker, 1947, p. 102). Furthermore, since it is known that large individuals of the same species have a relatively smaller surface area than do small ones, the larger size may be considered as an adaptation to an increase in the viscosity of the water which accompanies a decrease in temperature. Examples to support this assumption can be found in numerous papers, including those of Murray and Hjort (1912), and many students of Radiolaria, copepods and certain other forms (Hedgpeth, 1957). Kofoid (1930), for instance, reported that marine protozoa living in cold water grow to much larger sizes than do their relatives existing at higher temperatures.

Aquatic biology also offers many instances in which organisms of the same species grown at different temperatures may show a somewhat different shape. The phenomenon of cyclomorphosis, reviewed by Brooks (1946), is an example. In lamellibranch larvae, of course, no radical changes in structure, as observed in Daphnia populations, can be anticipated. Yet, it has been reported (Jørgensen, 1946, p. 296) that at least in some lamellibranchs, such as *Venus gallina*, "the larval outline varies from almost square to circular." According to the same author

the veligers of the common mussel, *Mytilus edulis*, of Danish waters also show a remarkable variability in their shape. The water temperature during development can again be suspected as a factor affecting the shape of the larva.

During the past few years larvae of approximately 20 species of lamellibranchs have been successfully cultured from fertilized egg through metamorphosis by members of our laboratory (Loosanoff, 1954). The data collected during these studies will soon permit us to offer reliable material for recognizing larvae of the species with which we have been working. It will include photomicrographs of the larvae and length-width measurements of their shells from early straight hinge stage until metamorphosis. However, before offering these criteria, it was deemed necessary to ascertain the following possibilities, which could reflect on the reliability of our material:

1) We wanted to know whether, as is suggested by some students, individuals of larval populations grown at relatively low temperatures reach a larger size before metamorphosing than do larvae grown in warmer water. If this is true, special corrections, perhaps as formulae, should be offered to show the relationship between average size at setting and water temperature.

2) Since one of the criteria for recognizing a larva nearing metamorphosis is its dimensions, *i.e.*, length and width, it was necessary to determine whether the length-width ratio is relatively constant or if it changes in conformance with the temperature of the water in which larvae develop.

The questions posed above could be answered only on the basis of well-controlled experiments in which the water temperature was the only factor varied. Obviously, because of the time and efforts required, it would have been difficult to conduct such experiments with larvae of all 20 species of lamellibranchs with which we were working. We decided, therefore, to limit ourselves to observations on one or two species only. This paper is devoted chiefly to a description of the observations on size and length-width ratio at the beginning of metamorphosis of larvae of the hard shell clam, *Venus* (*Mercenaria*) *mercenaria*, developing at different temperatures.

Certain aspects of the studies which provided data for this paper have already been described (Loosanoff, Miller and Smith, 1951). In brief, they consisted of growing larvae of *Venus* (*Mercenaria*) *mercenaria* at constant temperatures ranging from 15.0° to 33.0° C. at intervals of 3.0° C. Since fertilized eggs that were placed in water of 15.0° or 33.0° C. showed abnormal development and heavy mortality, few ever reaching veliger stage, growth of larvae at these temperatures will not be discussed here.

The work was done in winter, the time we find most convenient to control the water temperature (Loosanoff, 1949). Altogether, four experiments were conducted. However, in one experiment one of a pair of cultures grown at 24.0° C. was accidentally lost, while in the fourth experiment, which was conducted during a comparatively warm spell when low temperature was difficult to maintain, no cultures were carried at 18.° C. As is our practice, the water in the culture jars was changed every second day (Loosanoff and Davis, 1950). The larvae were fed a mixture of micro-organisms consisting principally of *Chlorella* sp.

Samples for larval measurements were taken 48 hours after fertilization and every second day thereafter, until metamorphosis. These samples consisted of 50 larvae measured at random from each culture vessel, *i.e.*, 100 larvae from each temperature group. The length represented the greatest distance between the anterior and

posterior shell margins, while the width was the distance measured from the tip of the umbo to the middle of the ventral shell margin.

Because the larvae could not be marked individually, their rate of growth and size at setting could not be recorded directly on this basis. For this reason we used two substitute criteria. One was the average length of the larvae on the day the setting was first observed in each culture, and the second, the maximum size of the larvae observed during the life of the culture.

The number of days required for setting to begin at the different temperatures in the four experiments is given in Figure 1. Clearly enough, there were differences between the cultures within the same temperature group and also between

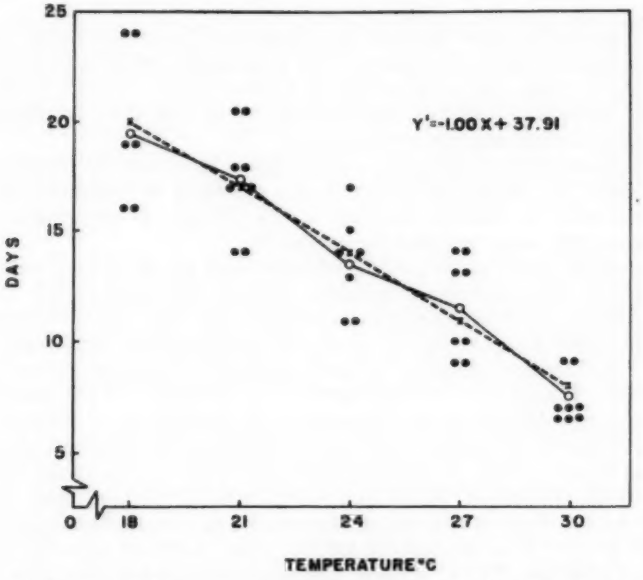

FIGURE 1. Number of days necessary for clam larvae to begin setting in cultures grown at different temperatures. ● = mean of individual culture; o = mean of all cultures grown at the same temperature; x = mean for given temperature predicted from regression line.

the groups carried at different temperatures. The most uniform results were obtained with the 30.0° C. group, where the beginning of setting in the different cultures varied between seven and nine days after fertilization, a difference of only two days. However, the difference between the shortest and longest periods needed for larvae to begin setting became greater in colder water. For example, in the 18.0° C. group the earliest beginning of setting was recorded 16 days after fertilization and the latest, after 24 days, a difference of eight days (Fig. 1).

An analysis of variance was carried out to test the significance of the differences among the different temperature groups on the number of days required for setting to begin. Since the result was highly significant (beyond the .001 level), separate

"t" tests were run for all possible pairs of temperature groups. All the "t" tests were highly significant (beyond the .01 level), with exception of the comparison between the 27.0° and 24.0° C. groups, and between the 21.0° and 18.0° C. groups. These results show, therefore, that a very strong relationship exists between temperature and date of setting, *i.e.*, larvae reared at high temperatures set significantly

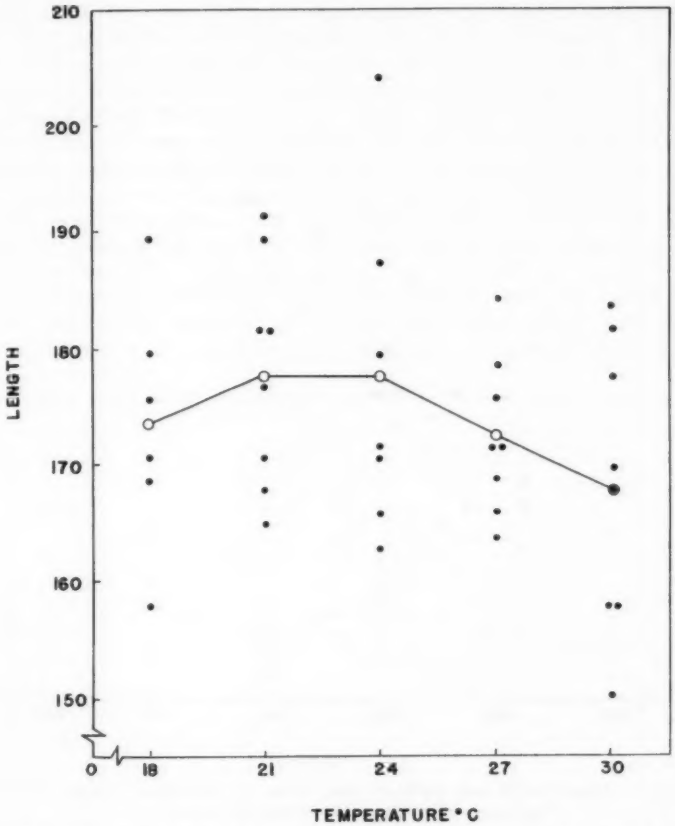

FIGURE 2. Mean length of clam larvae grown at different temperatures on day of beginning of setting. Measurements in microns. ● = mean of individual culture; o = mean of all cultures grown at the same temperature.

earlier than those raised at lower temperatures. This conclusion was expressed in the preliminary paper (Loosanoff, Miller and Smith, 1951).

Plotting of the dates of beginning of setting in the different cultures against the temperatures showed that the mean number of days for setting to begin for the various temperatures lies on an almost straight line (Fig. 1). Since the line con-

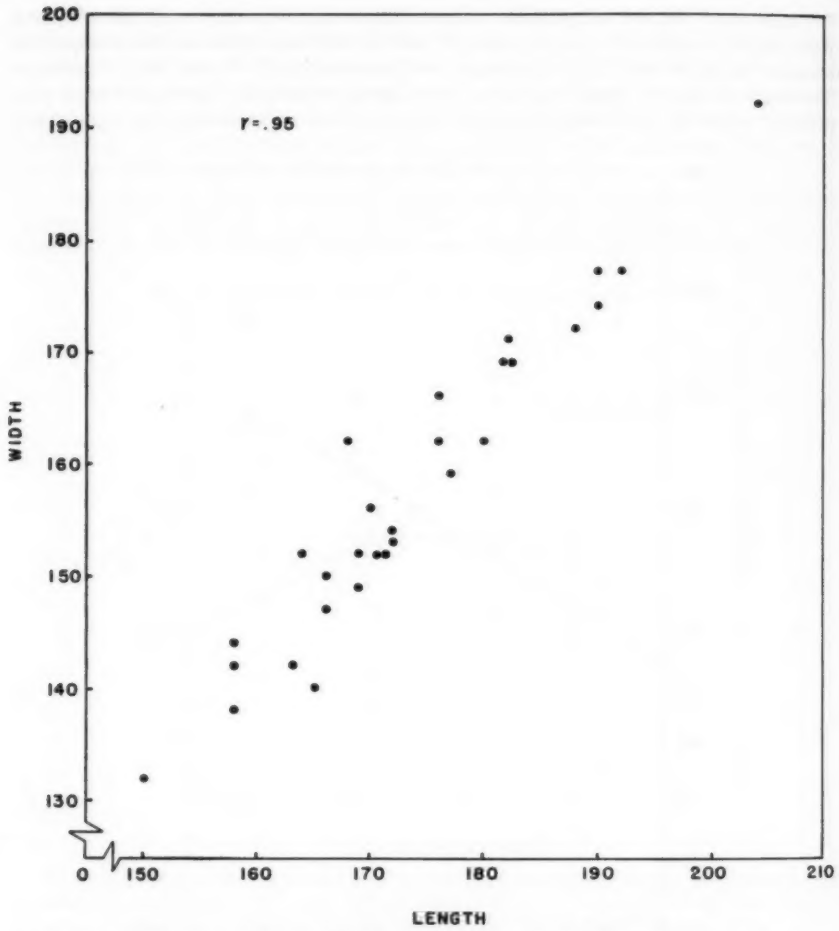

FIGURE 3. Mean length and width of clam larvae of individual cultures on day of beginning of setting. Measurements in microns.

necting the means of the different temperatures is obviously rectilinear, a regression equation was computed and found to be $Y' = -1.00\,X + 37.91$, where Y' is the predicted setting date and X is the temperature.

Because the regression line is an excellent fit to the experimental data, it is probable that interpolation within the 18.0° to 30.0° C. limits of the experiment can be made with a fair degree of confidence. However, extrapolation beyond these limits is not justifiable. This was shown by our other experiments, which demonstrated that clam eggs placed in water having a temperature of 15.0° or 33.0° C.

did not develop normally. Therefore, since the lineal regression does not hold even for a slightly higher or lower temperature, it cannot be expected to hold for lower or higher temperatures.

An analysis of variance test showed no significant differences among the five temperature groups, with respect to mean length of larvae at date of setting. Thus, although larvae grown at different temperatures required different periods to reach metamorphosis, in all cases they reach approximately the same mean length before setting. This observation indicates, therefore, that there was virtually no relationship between temperature and mean length at date of setting (Fig. 2). Nevertheless, the same figure shows that there was considerable variation in mean length at the beginning of setting among the various cultures within each temperature group.

In our studies we were also concerned with the shape, at metamorphosis, of larvae grown at different temperatures because, as has already been mentioned, the literature contains several remarks concerning variability of shape of larvae of the same species near setting time. Since the simplest method of describing the shape of a larva in mathematical terms for statistical analysis is to indicate its length-width ratio, measurements were made on larvae of all cultures, except those constituting the fourth experiment where no width measurements were taken, and the correlation between the mean length and the mean width of the larvae of each culture, on the day of the beginning of setting, was determined (Fig. 3). The

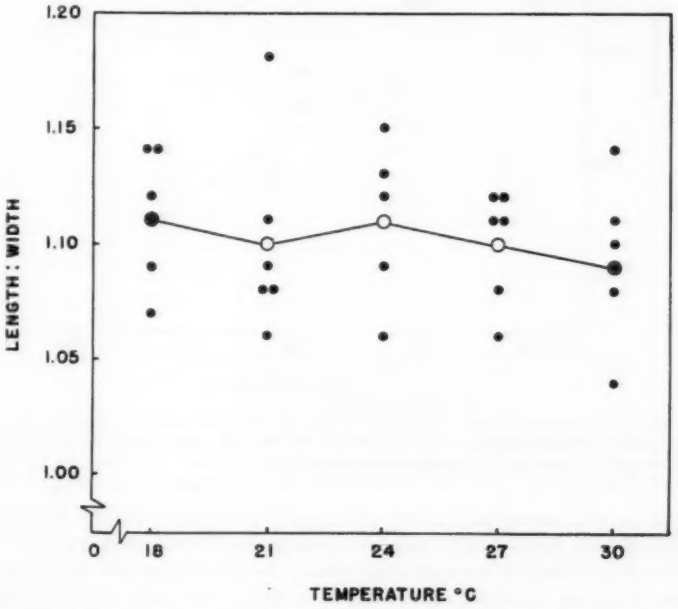

FIGURE 4. Ratio of mean length to mean width of clam larvae of different cultures on days of beginning of setting, in relation to temperature. ● = mean of individual culture; o = mean of all cultures grown at the same temperature.

results indicated this correlation to be so high ($r = .95$) that it seems unlikely that any analysis made using width as a variable would add anything new to that already made with length.

Continuing the analysis of data that might help in discovering the differences in shape of larvae grown at different temperatures, a study was made of the ratio of mean length to mean width at the date of setting. It failed to show the existence of any significant change in the ratio at different temperatures (Fig. 4). Thus, this matter has been satisfactorily solved to assure investigators working with lamelli-

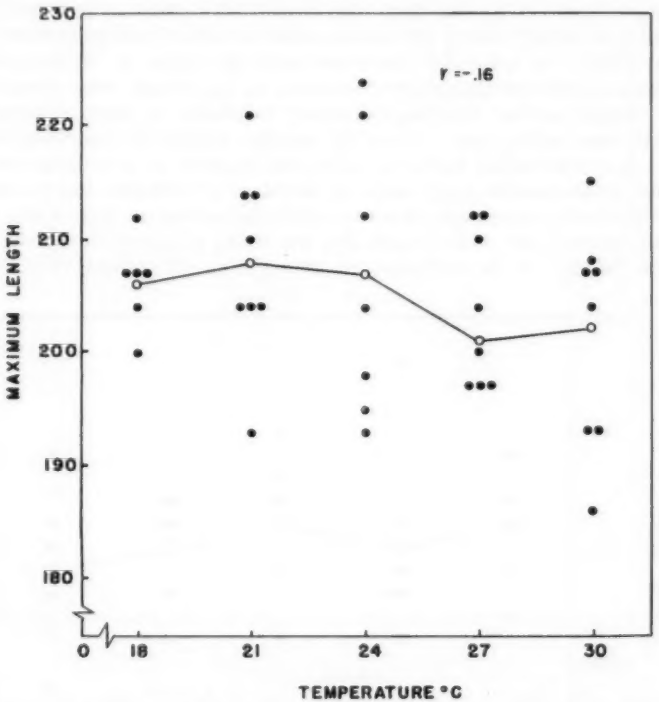

FIGURE 5. Maximum length of clam larvae of different cultures during the life of a culture, in relation to temperature. Measurements in microns. ● = mean of individual culture; o = mean for all cultures grown at the same temperature.

branch larvae that individuals of the same species display virtually the same shape at metamorphosis, even though they are grown at different temperatures.

Although our studies showed that even if larvae are grown at different temperatures, they, in all cases, reach approximately the same mean length before setting, the question still unanswered is whether there is an appreciable relationship between the *maximum* length of larvae on the date of setting and the temperature. A statistical analysis demonstrated the lack of an appreciable relationship

between these two variables, giving a correlation of $-.16$. The lack of relationship is clearly indicated in Figure 5, which shows that the means of the cultures grown at five different temperatures varied from 201 μ to 208 μ, a range of only seven microns. Nevertheless, we again noticed a considerable variability among the cultures within each temperature group, although it was much less pronounced within the 18.0° C. group than in certain others. However, again, no definite trend in this respect was observed because the variability of the maximum length of the

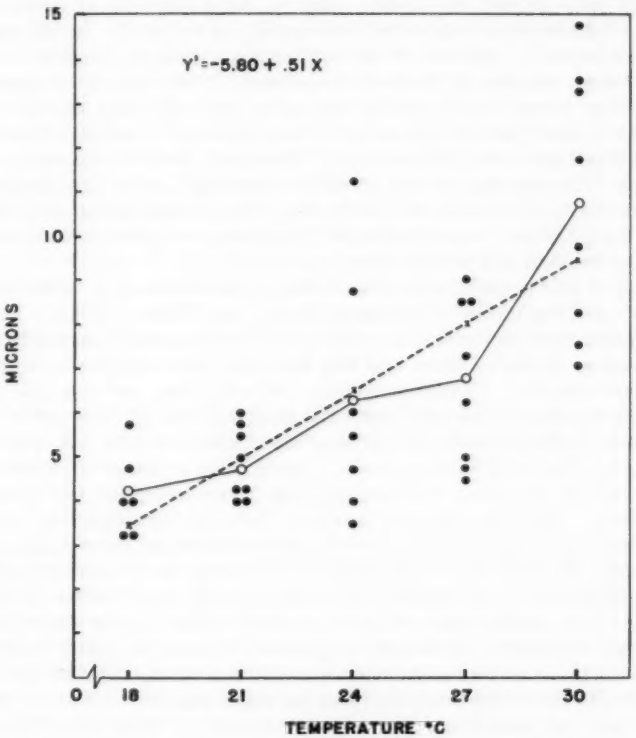

FIGURE 6. Average daily length increment of larvae grown at different temperatures. Measurements in microns. ● = mean of individual culture; o = mean of all cultures grown at the same temperature; x = mean for given temperature predicted from regression line.

larvae in the different cultures grown at 27.0° C., the second highest temperature group, did not differ greatly from that recorded for the lowest, *i.e.*, the 18.0° C. group.

Using the data collected during these studies, we can calculate the average daily growth increment for all cultures at given temperatures. By plotting the average daily growth increment for each temperature group against the corresponding

temperature, an approximately rectilinear relationship becomes evident (Fig. 6). The regression equation computed was found to be $Y' = -5.80 + .51\ X$, where Y' is the predicted average daily growth increment and X is the temperature. The relationship indicates, naturally, that faster growth occurred at higher temperatures and, theoretically, from the regression equation it can be assumed that if the temperature were reduced to about 11.3° C., growth would stop completely.

In discussing our results it must be remembered that they are representative only for this series of experiments and that there are many factors which can change the daily growth increment. One of these, capable of lowering or increasing the rate of growth of larvae, is the quality of the food. In our experiments the larvae were fed a mixture of phytoplankton, consisting largely of *Chlorella*. However, recent studies of Davis and Guillard (1958) clearly showed that the growth of clam larvae varied greatly depending upon the kind of food they were given. Davis found that the larvae grew best when fed a culture of mixed flagellates, including *Isochrysis*, *Monochrysis*, *Dunaliella*, and *Platymonas*, while the larvae given *Chlorella*, two species of which were tried, grew considerably slower. In the same series of experiments Davis was able to demonstrate that the rate of growth of larvae of the American oyster, *Crassostrea virginica*, also varied greatly according to the kind of food organisms available.

Quantity of food organisms is another factor to be considered. Our earlier work (Loosanoff and Engle, 1947; Loosanoff, Davis and Chanley, 1953a) showed that heavy concentrations of food cells, such as *Chlorella*, usually seriously interfered with the feeding of adult oysters and that they also either killed the clam larvae or retarded their growth. Furthermore, they indicated that the optimum concentration of food organisms depended upon the kind and size of their cells. Since, in our experiments described in this article, the number of cells was not accurately determined and the food did not consist of pure cultures but of a mixture of many organisms, we do not know whether the clam larvae were fed the optimum food concentrations. This circumstance, however, does not invalidate our comparisons because all cultures were given food of the same quality and in the same quantity.

Finally, the effect of the concentration of larvae in the experimental cultures should be considered. Our studies (Loosanoff, Davis and Chanley, 1953b; Loosanoff, 1954) have shown that larvae in crowded cultures grow somewhat slower. However, the difference in the rate of growth of larvae in lightly-populated and those in densely overcrowded cultures was not too great. For example, we determined, at the end of the tenth day, that the mean length of larvae in the cultures containing only six individuals per cubic centimeter of water was 162 μ, whereas the mean length in the overcrowded cultures containing 52 individuals per cubic centimeter was 144 μ, or only 18 μ less than that recorded for lightly-populated cultures. Since, in the experiments described here, we began with the same number of larvae in all containers and because during the experiments no excessive mortality was recorded in any of the cultures, our larval populations in all jars were not much different from each other and, therefore, could not seriously affect the uniformity of the experimental conditions.

In concluding this article a brief reference to one more aspect of the role of water temperature on growth of bivalve larvae may be appropriate. It has frequently been reported that species living in warmer water have just as long a pelagic life as their

northern relatives, and that, at a given temperature, the eggs of the southern species cleave and develop more slowly than those of the northern species of the same genus (Fox, 1936; Thorson, 1950). This suggests that even if the eggs and larvae were cultured under identical conditions, development of the eggs and larvae of the southern clam, *Venus* (*Mercenaria*) *campechiensis*, would require a longer period than is needed for eggs and larvae of the northern clam, *Venus* (*Mercenaria*) *mercenaria*. I had the opportunity to verify this contention by the studies conducted together with my associate, H. C. Davis. Adult *Venus* (*Mercenaria*) *campechiensis* were imported from the Apalachicola area of the Gulf of Mexico in November, 1953. Several weeks later these clams were conditioned for spawning. A group of large *Venus* (*Mercenaria*) *mercenaria*, natives of Long Island Sound, were ripened under identical conditions simultaneously with the southern species. When both groups were ripe, spawning was induced by our usual methods (Loosanoff and Davis, 1950). Fertilized eggs of each species and, later, larvae developing from these eggs were cultured under identical conditions, the temperature being approximately 21.0° C. Triplicate cultures of each species were grown, and random samples of 100 larvae from each culture were measured every second day. The curves constructed on the basis of this information showed that the rates of growth of the larvae of the two species were practically identical. Moreover, setting of larvae of both groups began at the same time. The results of this experiment contradict, therefore, the conclusion that when grown at the same temperature the eggs and larvae of the southern species develop more slowly than those of the northern species of the same genus.

I wish to express my thanks to Mrs. Barbara Myers for the statistical analysis of the data and to my associates, Miss Rita S. Riccio and Harry C. Davis, for their help in preparation of this article.

Summary

1. The mean setting dates for larvae of *Venus* (*Mercenaria*) *mercenaria* grown at constant temperatures of 30.0°, 27.0°, 24.0°, 21.0° and 18.0° C. were found to lie on an almost perfectly straight line according to equation $Y' = -1.00X + 37.91$, where Y' is the predicted setting date and X is the temperature.

2. There were no significant differences among the five temperature groups with respect to mean length of larvae at time of setting.

3. There was no apparent relationship between *maximum* length of larvae at time of setting and temperature.

4. The correlation between mean width and mean length of larvae at time of setting was very high ($r = .95$).

5. No apparent relationship was found between shape of larvae (*i.e.*, ratio of mean length to mean width) at time of setting and temperature.

6. The average daily growth increment for all cultures at given temperatures under the conditions prevailing during the experiments was determined.

7. The rate of growth of larvae of the southern clam, *Venus* (*Mercenaria*) *campechiensis*, was the same as that of the northern species, *Venus* (*Mercenaria*) *mercenaria*, when the temperature and other conditions were identical. Moreover, setting of larvae of both species began at the same time.

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ANTIGENIC DIFFERENCES BETWEEN STEM AND HYDRANTH IN TUBULARIA [1]

JOHN B. MORRILL, JR.[2,3]

Oceanographic Institute, Florida State University, Tallahassee, Florida

The marine hydroid, *Tubularia crocea*, consists essentially of two layers of tissue which are differentiated into a hydranth with hypostome, two groups of tentacles and gonophores, and a stem portion surrounded by a chitinous perisarc. Chemical differences may underlie these morphological differences between the hydranth and stem regions. Since the morphallactic regeneration of a hydranth from the stem region involves a remodeling of a certain portion of the stem tissues, further chemical changes may accompany visible morphological changes during regeneration of a hydranth. Because proteins and other complex molecules are the principal substances of organic forms, basic changes in morphogenesis probably involve production and transformation of such molecules and their aggregates.

The key role presumably played by the metabolism of complex molecules and especially proteins has interested developmental biologists for some time. A number of workers have used immunological methods to study differentiation of such substances and to correlate changes in their composition with visible morphological differentiation [reviewed by Irwin (1949), Ebert (1955), Tyler (1955, 1957), Woerdeman (1955), Schechtman (1955), Nace (1955), and Brachet (1957)]. While there have been many immunochemical studies concerned with embryonic development, immunological methods have rarely been used in studying problems of regeneration although the usefulness of such methods in studies on regeneration has been mentioned by Woerdeman (1953). De Haan (1956) and Laufer (1957) have used immunological techniques to study muscle differentiation in the regenerating limb of axolotl larvae.

In spite of numerous investigations on regeneration in *Tubularia* there is little information on the chemistry of this organism. A rational prerequisite to the investigation of chemical changes during hydranth regeneration would seem to involve the determination of chemical differences between the two main regions, the hydranth and the stem. Accordingly, the present study was undertaken to determine the similarities and differences in the antigenic composition of *Tubularia crocea* hydranths and stems (*cf.* preliminary note by Morrill, 1958).

Materials and Methods

A species of *Tubularia*, *T. crocea*, was collected from the St. John's River jetties in Florida, where the organism is abundant from October to June. Only

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[2] The major portion of this study was performed during tenure of a National Science Foundation Predoctoral Fellowship.

[3] Present address: Department of Biology, Wesleyan University, Middletown, Connecticut.

colonies whose stems were relatively free of macroscopic epizooites were used. After their collection the animals were kept in jars of sea water for not more than 36 hours.

Clean whole animals, stems, and hydranths were frozen in a dry-ice bath. Hydranths and stems were prepared by first cutting off the hydranths and then removing the distal 4 millimeters of the stem plus any basal region of the stem that had epizooites. Stems were frozen within 20 minutes after hydranth removal. Hydranths were frozen within 4 hours after they had been severed from the stems. Separation of hydranths and stems on a sexual basis proved to be impractical. The frozen tissues were lyophilized and stored at − 20° C.

Preparation of saline extracts for use as antigens

One-gram quantities of lyophilized tissue were ground in a mortar and extracted with 10 ml. of buffered saline (9 g. NaCl/l., 0.1 *M* phosphate buffer, pH 7.0) for 8 to 12 hours at 4 to 10° C. At the end of this period the supension was homogenized in a glass homogenizer in an ice bath. The homogenate was kept at 4 to 10° C. for two more hours. Then the homogenate was centrifuged for 30 minutes at a centrifugal pressure of approximately 4500 *g* and the cloudy supernatant collected and stored at − 20° C. The nitrogen content of the extracts, determined by a modified nesselerization method (Hawk *et al.*, 1954), varied as follows: whole animal, 0.15 to 0.50 mg. N/ml.; hydranth, 0.25 to 0.82 mg. N/ml.; stem, 0.23 to 0.68 mg. N/ml.

The degree of contamination with insoluble particulate matter may have varied in the extracts. Therefore, equilibration on a nitrogen basis is at best an approximate equalization of antigen concentration.

Preparation of antisera

Nine male rabbits were used in the preparation of antisera. None of the preinjection rabbit sera precipitated the extracts. Several injection routes were employed. These included intravenous, intermuscular with oil emulsion adjuvant (Freund, 1947), and intraperitoneal injections. The intraperitoneal route yielded the most satisfactory antisera. Two rabbits received three series of intraperitoneal injections over a two-month period. Each series consisted of 10 to 15 milliliters of whole animal extract administered within 6 to 8 days. The extracts injected into these rabbits were not adjusted on a nitrogen basis. One of these rabbits (Rabbit 4) was injected with the supernatant fraction of the homogenate. The other (Rabbit 6) was injected with the entire homogenate.

Blood was collected in sterile 50-milliliter centrifuge tubes 5, 7, and 17 days after the last injection of each series and allowed to clot at room temperature for 4 to 5 hours. The clots were loosened and the tubes stored at 4 to 10° C. for 24 hours. The serum then was decanted, centrifuged, and stored in vials at − 20° C. until used.

Serological tests

In order to test for antigenic differences between hydranth and stem tissues two types of precipitin tests were used. The first test employed was the standard interfacial ring test. When this test failed to reveal any antigenic differences, even when antiserum absorbed with hydranth or stem extract was used, the Ouchterlony

agar gel diffusion method was employed in order to determine the spectrum of individual precipitin reactions and what differences might exist between the spectra of precipitin lines produced by anti-whole animal serum and stem and hydranth extracts.

Interfacial ring tests were performed by layering 0.05 ml. *Tubularia* extract over 0.05 ml. antiserum or over saline or pre-injection serum controls in 3 × 20 mm. capillary tubes. The tubes were capped with plastocene clay, incubated at 37° C., and examined after one and two hours for the presence of a precipitating ring at the interfaces. The highest antiserum titer (dilution of antiserum) of the sera from the two rabbits was 64. The highest antigen titer of the saline extracts was 16,000. These titers were obtained with the sera collected 5 and 7 days after the third injection series. No precipitates formed in control tests where pre-injection serum plus saline extracts and antiserum plus saline alone were employed.

The agar gel diffusion technique of Ouchterlony (Ouchterlony, 1949) was employed with modifications by Nace (personal communication). Details of the method are as follows. Four per cent agar was prepared and washed in distilled water for several days. This was used to prepare an aqueous 2 per cent agar containing aqueous merthiolate (0.25 ppth) and methyl orange (40 mg. per 500 ml. agar). A basal layer of this melted 2 per cent agar was poured on the bottom of petri dishes. After this layer had hardened additional agar was added and a lucite well mold placed in position. When the agar had hardened the mold was removed.

The wells were filled with antigens and antisera—0.30 ml. in the large square well and 0.15 ml. in the narrow rectangular wells. The center well of each plate was filled with antiserum or pre-injection serum and the four surrounding wells with saline extracts or saline. The plates were incubated in high humidity in an air-tight container at 37° C. for 7 days. They were then brought to room temperature for 6 to 24 hours and finally left at 5° C. for an additional 7 days.

At the end of 14 days the agar plates were fixed in 5 per cent formalin with methyl orange added, mounted between thin glass plates, and inserted into a photographic enlarger. The focused image of the wells and precipitin lines was so faint, particularly in the region of coalescence of lines, that photographing the preparations proved to be impractical. Therefore, the enlarged diagram of the lines was recorded by tracing on paper .

The antisera were absorbed in the following way: antisera and saline extracts or saline controls were mixed in various proportions in small sterile tubes, incubated at room temperature for two hours, placed in the refrigerator for 36 to 48 hours, and centrifuged. The supernatant was then subjected to interfacial ring tests and agar gel diffusion tests.

Results

In order to test for differences in the antigenic composition of *Tubularia* hydranths and stems three tests were employed—the precipitin ring test, the Ouchterlony agar gel diffusion test, and the absorption test which was used in conjunction with the other two tests.

Precipitin ring tests

Precipitin antigen titers were determined in order to test for quantitative and qualitative differences between stem and hydranth extracts. Such extracts were

equilibrated on a nitrogen basis, serially diluted and layered over anti-whole animal antiserum. No differences in the titers were observed. Evidently the two types of extracts were similar on a gross quantitative basis. To test the possibility of the existence of qualitative differences, absorption experiments were performed. Partial absorption of anti-whole animal antiserum by either extract produced antiserum which still reacted equally with both types of extract; complete absorption by either extract produced sera which failed to form a precipitin ring with either extract. The precipitin ring tests then failed to reveal any distinct antigenic differences between hydranth and stem extracts.

Because these tests failed to reveal any antigenic differences, it seemed desirable to utilize a method where the precipitate of the precipitin ring could be separated into a spectrum of one or more precipitin reactions. Accordingly, the antigenic composition of the extracts was examined by means of the agar gel diffusion technique of Ouchterlony.

Ouchterlony tests

Tests with unabsorbed sera. Preliminary Ouchterlony tests of anti-whole animal sera revealed multiplicity of precipitating systems. Similarities as well as differences existed between the precipitin patterns with stem and hydranth extracts. As expected, the number of lines formed varied with the antisera from the different rabbits. No precipitin lines appeared with pre-injection sera and saline controls. It was found that the pattern of precipitin lines varied in repeated tests with a given antiserum, possibly because the saline extracts used as test antigens were prepared at different times, even though the extracts were prepared by a standard procedure and adjusted on a nitrogen basis. In addition, antisera from a rabbit taken at different times following a series of injections exhibited variations in position and in number of lines in the precipitate patterns when tested with hydranth and stem extracts. This is probably because the maximum concentration of antibodies for the several antigens did not occur at the same time (Abramoff and Wolfe, 1956). The most complete precipitin line patterns were obtained with antisera from Rabbit 4 and Rabbit 6 (see under Methods) obtained 5 and 7 days after the third series of intraperitoneal injections.

The best precipitin pattern produced by anti-whole animal sera with hydranth and stem extracts is given in Figure 1. This antiserum from Rabbit 4 produced a total of seven precipitin lines with hydranth extract and seven precipitin lines with stem extract. Six precipitin lines with hydranth extract coalesced with five lines produced with stem extract. One additional line was restricted to the hydranth extract. Two precipitin lines and a spur on a coalescing line were restricted to the stem extract. Fewer lines were produced with antiserum of Rabbit 6. In the best pattern with antiserum from this rabbit four lines produced by hydranth extract coalesced with three lines formed by hydranth extract. In addition two precipitin lines were limited to reactions with components of stem extract, and possibly one was limited to hydranth extract.

In the several experiments the coalescing lines formed complex patterns. In addition to the fusion of single lines formed by the two extracts, there were instances where two hydranth precipitin lines coalesced with one line formed by stem extract and vice versa. These results may be interpreted as being due to superimposed

precipitin lines in the reaction of the antiserum with one extract. It is also possible that in one extract an antigenic substance had haptens in common with the haptens of two antigenic substances in the other extract (Kaminski and Ouchterlony, 1951). In nearly all the experiments one of the stem lines that coalesced with a hydranth line had a spur which extended beyond the region of coalescence (Fig. 1). This indicates that this stem antigen had two haptens, one in common with a hydranth antigen and one not found on any of the hydranth antigens. This interpretation is in accord with the explanation for the appearance of spurs given by Kaminski and Ouchterlony (1951).

The large number of lines formed in the Ouchterlony patterns suggested that some of the lines might be due to excessive quantities of certain antigens or antibodies (Kaminski, 1954). It was also possible that one or more of the single lines might be due to several superimposed precipitates (Grasset *et al.*, 1956). Attempts to resolve the complexity of the patterns, however, by diluting either the antisera or

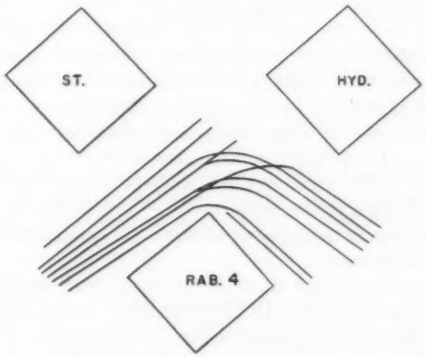

FIGURE 1. Diagram of precipitin pattern of anti-whole animal serum, Rabbit 4, with stem and hydranth saline extracts.

the hydranth and stem extracts resulted in patterns with fewer and more diffuse lines.

The number of lines was also checked by the immunoelectrophoretic method of Grabar and Williams (Grabar and Williams, 1953, 1955). In the first experiment the antigens of whole animal extract were separated in 2 per cent agar electrophoretically (veronal buffer, pH 8.6, ionic strength 0.1, 10 ma., 24 hrs.). After the separation, anti-whole animal serum of Rabbit 4 was placed in a trench parallel to the path of migration and allowed to diffuse into the agar. After seven days' incubation, nine precipitin lines in the form of distinct arcs were detected. In another experiment extracts of hydranths and stems were separated electrophoretically (veronal buffer, pH 8.6, ionic strength 0.05, 30 ma., 5 hrs.) and tested with anti-hydranth serum. This antiserum produced six curving precipitin lines with each extract. The electrophoretic mobilities of the several arcs were similar. This particular antiserum when previously tested by the Ouchterlony method had produced six lines with hydranth extract and five lines with stem extract.

The patterns formed by coalescing lines in the Ouchterlony tests demonstrate a number of antigens to be common to both hydranth and stem. The immunoelectrophoretic patterns showed that at least six of the hydranth antigens had electrophoretic mobilities comparable to those of six stem antigens. In addition to the similar antigens the tests with unabsorbed sera showed one antigen to be limited to the hydranth and at least two antigens limited to the stem.

Tests with absorbed anti-whole animal serum. In order to resolve further the antigenic differences between hydranth and stem extracts, absorbed serum (Rabbit 6) was used in Ouchterlony tests. Antiserum absorbed with hydranth extract still produced two distinct and one faint, diffuse precipitin lines with stem extracts. Antiserum absorbed with stem extract produced one precipitin line with hydranth extract. The absorbed sera failed to produce bands with the absorbing antigen. These results again indicate there are at least two antigenic substances restricted to stems and one restricted to hydranths.

Discussion

The results demonstrate that precipitating antibodies can be obtained against saline extracts of *Tubularia*. The agar diffusion tests show that a spectrum of precipitating antigens is present in stems and hydranths.

It is possible that some of these antigenic substances are not actually part of the tubularian tissues. In spite of the precautions described (see methods section), the antigens restricted to the stem extracts may be from organisms associated with the perisarc which is limited to the stem region. The antigenic differences may also be due to breakdown products of ingested food. The discussion presented here is subject to these reservations.

A number of antigens appear to be common to both hydranths and stems. It was previously reported (Morrill, 1958) that there were seven common antigens. Re-examination of Ouchterlony patterns has revealed that at least three antigens are common to both regions of the animal. In addition, one stem antigen has antigenic sites similar to those on two hydranth antigens. Immunoelectrophoretic experiments with anti-hydranth serum showed six antigens of hydranths and stems to have similar electrophoretic mobilities. This method resulted in distinct non-overlapping lines and should prove useful in future studies on the antigenic composition of this organism. Ouchterlony tests with non-absorbed and absorbed anti-whole animal sera indicate that at least two antigenic substances are limited to stems and one to hydranths.

With the establishment of antigenic relations between stems and mature hydranths further investigations need to be conducted to determine the antigenic relations between stems, regenerated hydranths, and hydranths at different stages of regeneration. The antigens need also to be characterized. Preliminary experiments show that antisera inhibit hydranth regeneration. Hydranth-specific and stem-specific antibodies should now be tested for inhibiting action on the regeneration of hydranths.

I wish to express my appreciation to Dr. Charles B. Metz for his guidance, encouragement, and interest during the course of this investigation.

Summary

1. The antigenic composition of hydranths and stems of *Tubularia crocea* has been studied by means of the precipitin ring tests, Ouchterlony agar gel diffusion tests, and immunoelectrophoresis.

2. Precipitin ring tests showed that antiserum against whole animals contained precipitating antibodies but failed to reveal antigenic differences between hydranths and stems.

3. Ouchterlony tests of anti-whole animal serum and saline extracts of hydranth and stem tissues revealed the following:

a. Four antigens common to both regions of the animal.
b. One stem antigen with hapten sites similar to those on two hydranth antigens.
c. Two antigenic substances limited to stems.
d. One antigenic substance limited to hydranths.
e. One stem antigen with at least two haptens—one in common with a hydranth antigen and one which was not related to any precipitating hydranth antigens.

4. Six stem antigens and six hydranth antigens had comparable electrophoretic mobilities.

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ADENOSINETRIPHOSPHATASE OF MYTILUS SPERMATOZOA. II. EFFECTS OF SULFHYDRYL REAGENTS, TEMPERATURE AND INORGANIC PYROPHOSPHATE[1]

LEONARD NELSON[2]

Department of Anatomy, University of Chicago, Chicago 37, Ill. and Marine Biological Laboratory, Woods Hole, Mass.

The enzyme concentrated in the sperm flagellum of the mollusc, *Mytilus edulis*, which splits adenosinetriphosphate (ATP) has been classified as a "true" adenosinetriphosphatase (ATP-ase) (Nelson, 1955). Mohri (1958) has confirmed this in studies on the flagellar ATP-ase of sea urchin sperm. This class of enzyme liberates only one phosphate group from each ATP molecule, even on prolonged incubation. The Mytilus sperm enzyme exhibits optimum activity at about pH 8.4 in veronal and glycine buffers, while for sea urchin sperm, pH 8.8 is optimum in Tris and veronal buffers. Magnesium has a pronounced activating effect on both the molluscan and the echinoderm sperm ATP-ase; however, calcium exerts considerably less activation, and, moreover, antagonizes the potentiation due to magnesium. Dilution of filtered-sea-water or isotonic KCl suspensions of Asterias and Mytilus sperm tails with large volumes of glass-distilled water or 10^{-4} *M* $MgCl_2$ causes slow precipitation of the tails; this may be accelerated by the addition of small amounts of ATP. However, extruded threads produced from these sperm tail suspensions do not contract; this may be attributed to the lack of continuity of the components—the individual sperm tails (unpublished observation). Salts of heavy metals and other sulfhydryl reagents which serve as spermicidal agents act to halt sperm motility and also interfere with a regulatory mechanism of sperm respiration; low concentrations of inhibitor permit "uncontrolled" acceleration of oxygen consumption, while higher concentrations completely stop O_2 uptake and motility (Barron *et al.*, 1948; MacLeod, 1951). It has also been observed that sodium pyrophosphate, apparently by forming firm complexes with the divalent cations essential for optimal activity, exerts an inhibitory action on sperm ATP-ase (unpublished). The first paper in this series dealt with some of the characteristics of the Mytilus sperm tail ATP-ase. The effect of some additional agents (SH-inhibitors, temperature, pyrophosphate) on spermatozoan ATP-ase is considered in the investigations reported here.

Materials and Methods

Since it is difficult to induce spawning in the Woods Hole Mytilus either by temperature shock or the injection of KCl, it was necessary to obtain the sperm from minces of the gonads. The sperm were harvested and prepared as reported

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[2] Present address: Department of Physiology, Emory University, Atlanta 22, Ga.

previously (Nelson, 1955). "Decapitation" was effected by means of a stainless steel, piston-type homogenizer, and the heads and tails separated by repeated gentle centrifugation (1000 × g for ten minutes each) in isotonic KCl. The sedimented heads were discarded. The dilution-precipitation effect was exploited in the further isolation and purification of the tails from the pooled supernatant fractions by the addition of at least five volumes of ice-cold 10^{-4} *M* $MgCl_2$. After standing in the refrigerator for 6 to 12 hours, the tails had settled out and the clear supernate was decanted and discarded. The flocculent precipitate was further concentrated by centrifugation at 7000 × g for 10 minutes. The sperm tail concentrate was then taken up in two to four volumes of isotonic (*M/2*) KCl. (All solutions were

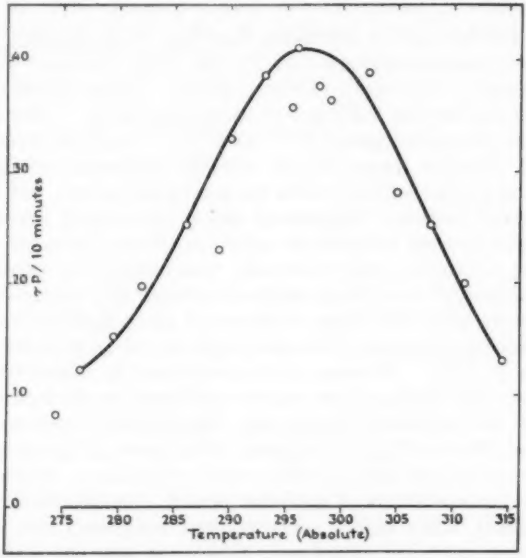

FIGURE 1. Temperature dependence of Mytilus sperm tail ATP-ase. Reaction mixture: 0.05 *M* KCl; 0.04 *M* Tris buffer, pH 8.6; 10^{-4} *M* $MgCl_2$; 10^{-3} *M* ATP; 0.1 ml. sperm tail suspension; total volume: 1.0 ml. Incubation time, 10 minutes. Ordinate, phosphate liberated in 10 minutes; abscissa, temperature, degrees, Absolute.

made up in de-ionized water.) Since the tail preparation resisted solubilization in mild alkaline and detergent solutions, no further effort was made to extract them and the experiments were performed on the "intact" tails. The reactants, mixed by lateral agitation in 12-ml. Pyrex conical centrifuge tubes, consisted of 0.1 or 0.2 ml. of sperm tail suspension, 0.8 or 0.7 ml. Tris buffer (Sigma), pH 8.6 (0.05 *M* KCl, 10^{-4} *M* $MgCl_2$, 0.04 *M* Tris), unless otherwise noted. After the reactants had equilibrated in a thermostat at 24° to 25° C., 0.1 ml. 10^{-3} *M* ATP (Sigma disodium salt, neutralized with NaOH to bromthymol blue endpoint) was added and the mixture allowed to incubate for ten minutes. Addition of one ml. of ice-cold 10% trichloroacetic acid terminated the reaction. The precipitate was

removed by centrifugation and the entire supernate analyzed for orthophosphate by the microcolorimetric method of Taussky and Shorr (1953). Optical density was measured in a Coleman, Junior spectrophotometer at a wave-length of 660 millimicrons. Protein content of the sperm tail samples was estimated by a modification of the method described by Nielsen (1958).

Results

Temperature-dependence of flagellar ATP-ase. Duplicate determinations of the enzyme activity at various temperatures over a range from 1° to 41.5° C. show a fairly constant increase in rate up to about 20° C., virtually a doubling for each

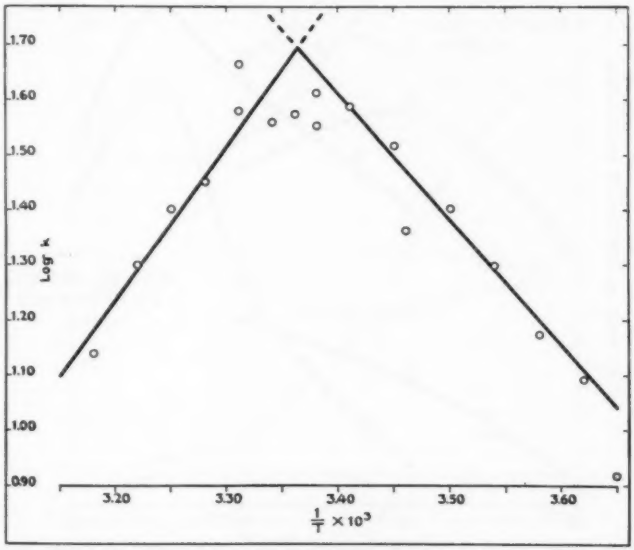

FIGURE 2. Temperature dependence of Mytilus sperm tail ATP-ase. Arrhenius plot of data in Figure 1. Ordinate, logarithm of rate of phosphate liberated; abscissa, reciprocal of temperature, 1/degrees, Absolute.

ten-degree rise ($Q_{10} = 2$) (Figure 1). Between 20° and 30°, the rate of dephosphorylation levels off and then declines fairly uniformly. From the slope of the Arrhenius plot (Fig. 2), the activation energy of the reaction was calculated to be − 10,450 cal./degree/mole. Since these determinations have been made on crude preparations, on "whole" tail suspensions, rather than purified enzyme extracts, this finding suggests that the broad temperature range of maximum enzyme action may simulate the situation which occurs during natural spawning. This coincidence may be of significance in that when associated with other factors (chemotactic, antigenic, etc.; *cf.* review by Rothschild, 1956) which may operate to assure maximum fertilization, optimum swimming activity of the spermatozoa may further serve

to increase the number of effective sperm "collisions" with activatable eggs. The dependence of spermatozoan motility on utilization of ATP has been established (Mann, 1945; Rothschild and Mann, 1950; Nelson, 1958a).

Effect of sodium pyrophosphate on sperm ATP-ase

Presence of an inorganic pyrophosphatase in flagella. Preliminary observations indicated that when the sperm tail incubation medium contained ATP and sodium pyrophosphate (NaPP) in the ratio of 1.6:1, the amount of inorganic phosphate

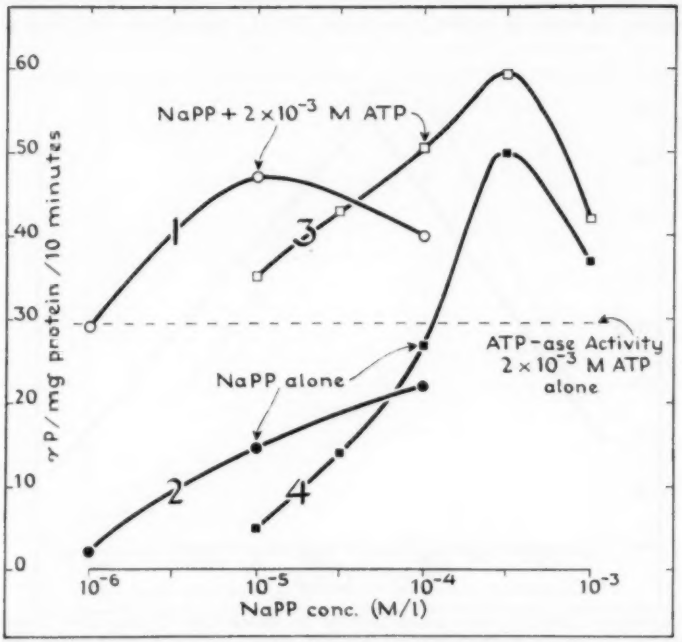

FIGURE 3. ATP-ase and IPP-ase activity of isolated Mytilus sperm tails. Reaction mixture: 0.05 *M* KCl; 0.04 Tris buffer, pH 8.6; 5×10^{-6} *M* $MgCl_2$ (dotted line and Curve 1, open circles, and Curve 2, closed circles) or 10^{-4} *M* $MgCl_2$ (Curve 3, open squares and Curve 4, closed squares); 2×10^{-3} *M* ATP (Curves 1 and 3 and dotted line); varying concentrations of NaPP; 0.1 ml. sperm tail suspension. Total volume, 1.0 ml. Incubation: 10 minutes, 24.5° C. Ordinate, γ phosphate liberated/mg. protein/10 minutes, abscissa NaPP concentration (M/liter).

liberated was 88% of the control (no NaPP). When the molar ratio of ATP to NaPP was 0.8:1, only 57% of the control activity was found. Tentatively, this was interpreted as an inhibition caused by the removal of the activating divalent cations through their chelation by the pyrophosphate. Verification was deferred until the present.

The sperm tail suspension, incubated for 10 minutes at 24.5° C. in 2×10^{-3} *M*

ATP and 5×10^{-5} *M* $MgCl_2$, splits off about 30 γ phosphate per mg. of sperm tail protein (dashed line, Fig. 3). When varying amounts of NaPP are added to this mixture, the increase in phosphate liberation evidently depends on the relative concentrations of NaPP and $MgCl_2$ in the medium. Curve 1 (open circles) shows that a peak of activity is reached at 10^{-5} *M* NaPP in the ATP-containing medium; while at a somewhat higher $MgCl_2$ (10^{-4} *M*) concentration, curves 3 and 4 (open

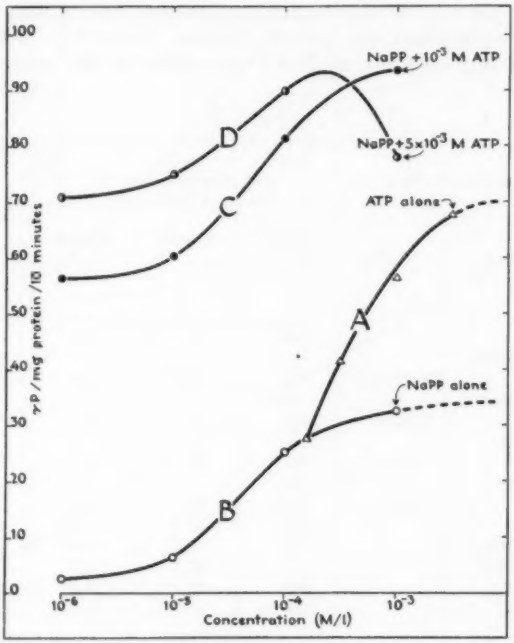

FIGURE 4. ATP-ase and IPP-ase activity of isolated Mytilus sperm tails. Reaction mixture: 0.05 *M* KCl; 0.04 *M* Tris buffer, pH 8.6; 5×10^{-5} *M* $MgCl_2$; varying concentration of NaPP (Curves B, C, D, open, closed and half circles, respectively); varying concentrations of ATP (Curve A, open triangles); 10^{-3} *M* ATP (Curve C), 5×10^{-3} *M* ATP (Curve D); 0.1 ml. sperm tail suspension (more concentrated than in previous figures). Total volume 1.0 ml. Incubation: 10 minutes, 24° C. Ordinate, γ phosphate liberated/mg. protein/10 minutes. Abscissa, concentration of phosphate ester (NaPP—curves B, C, D; ATP—curve A).

and closed squares) exhibit peaks of activity at 5×10^{-4} *M* NaPP, both in the presence and absence of ATP .

It is apparent from these results that (i) the sperm tail preparations exhibit an active inorganic pyrophosphatase (IPP-ase); (ii) that this enzyme activity is additive to that of the ATP-ase; (iii) that above an optimum substrate (NaPP) concentration there is a depression of the IPP-ase; (iv) that this optimum may be related to the magnesium concentration; and (v) that the presence of ATP may even aggravate the depression of IPP-ase activity under certain circumstances.

These observations were confirmed and the situation elucidated with a fresh sperm tail preparation. The conditions of the experiment were adjusted by doubling the $MgCl_2$ concentration (from 5×10^{-5} *M* to 10^{-4} *M* $MgCl_2$), by making up the ATP in the Tris buffer-KCl-$MgCl_2$ medium to maintain the total [$MgCl_2$] constant while varying the [ATP] and by increasing the sperm tail content in the incubation mixture (Fig. 4). The ATP-ase activity curve (A) approaches a maximum velocity of 70 γ phosphate/mg. protein/10 min. with increasing substrate concentration. The IPP-ase activity approaches a maximum velocity about half that of the ATP-ase (34.5 γ phosphate/mg./protein/10 min., Curve B). When, as shown in Curve C, increasing amounts of NaPP are added to the sperm tail incubation

TABLE I

1 Substrate concentration (M/liter)			2 Phosphate liberated (γ)			3 Difference	
ATP	NaPP	ATP + NaPP	Observed or extrapolated	If ATP	If NaPP	γ Phosphate	
			a	b	c	a − b	a − c
0.001			56	56	32.5	0	23.5
	0.000001		2.5				
		0.001001	56	56.5	32.5	−0.5	23.5
	0.00001		6.5				
		0.00101	60	57	32.5	3	27.5
	0.0001		25				
		0.0011	81	60	33	21	48
	0.001		32.5				
		0.002	93.5	62	33.2	31.5	60.3
0.005			67.5	67.5	34.5*	0	33
	0.005001		71	67.5	34.5*	3.5	36.5
	0.00501		75	67.5	34.5*	7.5	41.5
	0.0051		90	68	34.5*	22	55.5
	0.006		78	69*	34.5*	9	43.5

* V_{max} calculated from Lineweaver-Burk plot (Fig. 5).

ATP-ase and IPP-ase activity. Reaction mixture: 0.05 *M* KCl, 0.04 *M* Tris, pH 8.6, 5×10^{-5} *M* $MgCl_2$; 0.1 ml. sperm suspension; and varying concentrations of ATP and NaPP. Total volume, 1.0 ml. Incubation conditions: 10 minutes, 24.5° C. Enzyme activity = γ phosphate split/mg. protein/10 minutes incubation.

medium containing 10^{-3} *M* ATP, the activity is increased by 50 to 60 γ phosphate at all concentrations of NaPP, so that activity curves B and C appear parallel. However, when the medium contains 5×10^{-3} *M* ATP plus increments of NaPP (Curve D), the initial increase in rate of dephosphorylation approximates that attributable to the increase in ATP concentrations. Subsequently the rate increases slowly, approaching the maximum attained in Curve C, although at about 1/5 the NaPP concentration. Thereafter the velocity of the enzymic action declines rapidly.

The data summarized in Table I support the conclusion that two distinct enzymes are involved in the dephosphorylation of ATP and NaPP. If all the hy-

drolyzable phosphate were present as ATP, in Curve C of Figure 4, the maximum amount of phosphate split under the conditions of this experiment (enzyme concentration limiting) could not greatly exceed 62 γ instead of 93.5 γ (column 2); if the total source of P were NaPP, then the maximum P liberated probably could be only 33.2 γ. Similarly, if total hydrolyzable P in Curve D were present in the

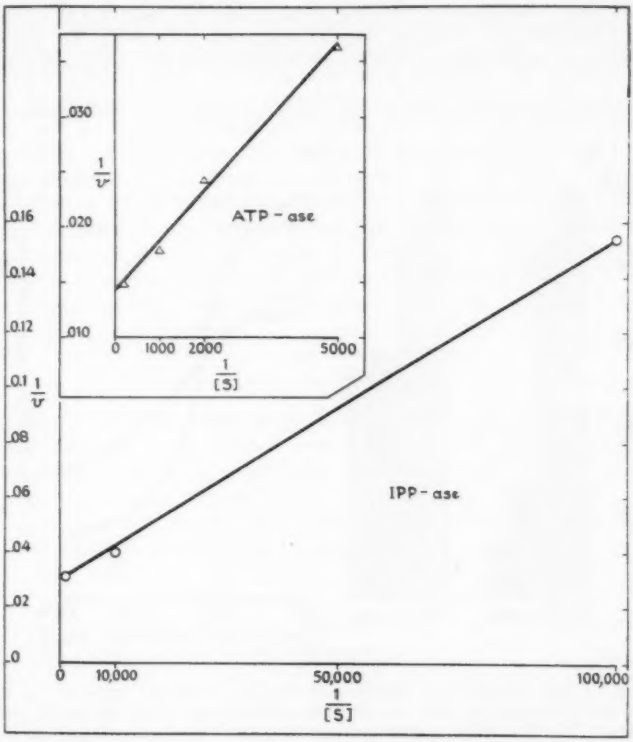

FIGURE 5. ATP-ase and IPP-ase activity of isolated Mytilus sperm tails. Lineweaver-Burk plot. Reaction mixture: 0.05 *M* KCl; 0.04 *M* Tris buffer, pH 8.6; 5×10^{-5} *M* $MgCl_2$, 0.1 ml. sperm tail suspension; varying concentrations of substrate; total 1.0 ml. Incubation: 10 minutes, 24° C. Open circles—IPP-ase; inset-open triangles—ATP-ase. Ordinate, reciprocal rate of phosphate liberation/mg. protein/10 minutes; abscissa, reciprocal of substrate concentration. $K_{M\text{-IPP-ase}} = 4.3 \times 10^{-5}$; $V_{max\text{-IPP-ase}} = 34.5$. $K_{M\text{-ATP-ase}} = 3.1 \times 10^{-4}$; $V_{max\text{-ATP-ase}} = 70$.

form of ATP, the maximum liberated P would not exceed 69 γ, or if present in the form of NaPP, probably would not be in excess of 34.5 γ. It is further evident that when both sperm tail content and $MgCl_2$ concentration in the incubation mixture are increased, in contrast to the situation in Figure 2, the NaPP does not interfere with itself as suggested by the optimum in curve 4, Figure 2, but that addition of

ATP at high enough concentration duplicates this phenomenon (Curve D, Fig. 3). Mohri (1958) and Nelson (1955) have shown the distinctive magnesium activation of the sperm tail ATP-ase. Evidence from the literature suggests that, with one notable exception cited in Discussion, regardless of enzyme source, inorganic pyrophosphatase activity is generally limited by the magnesium content of the medium. In this respect, the flagellar IPP-ase is not unique. The mutual dependence of these enzymes on adequate magnesium ion suggests the validity of the original concept that by chelation, NaPP competes with ATP for the magnesium ions and thereby could exert an inhibitory influence on the ATP-ase activity. The

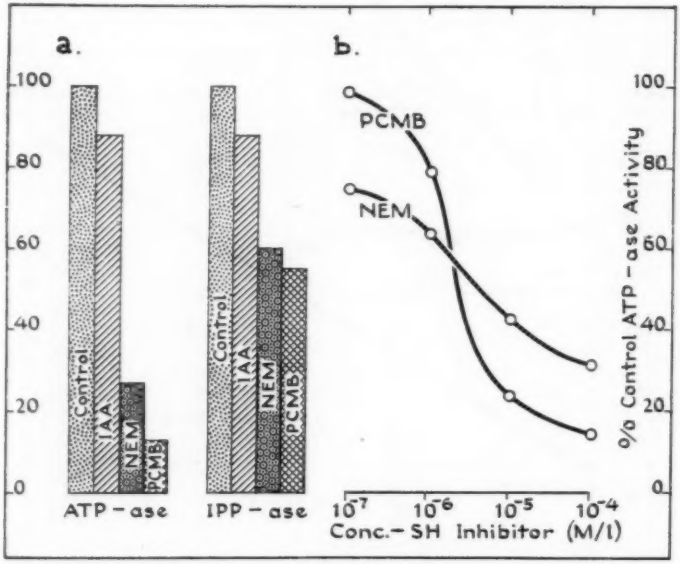

FIGURE 6. Effect of sulfhydryl inhibitors on Mytilus sperm tail enzymes. a. Effect of 10^{-4} *M* SH-inhibitor: Ordinate, per cent of control enzyme activity; abscissa, bar 1, control; bar 2, monoiodoacetate; bar 3, N-ethyl maleimide; bar 4, p-chloromercuribenzoate. b. Curves showing effect of varying concentrations of N-ethyl maleimide and p-chloromercuribenzoate on sperm tail ATP-ase. Ordinate, per cent of control ATP-ase (no inhibitor); abscissa, concentration of SH-inhibitor (*M*/liter). Incubation: 10 minutes, 24.5° C.

possible significance for sperm motility of this mutual interaction of substrates and enzyme activities will be considered later.

When the Lineweaver-Burk (1934) analysis is applied to these unpurified preparations, the Michaelis constants K_m, and the maximum velocities V_{max} of the respective enzymatic reactions may be determined graphically (Fig. 5). For the IPP-ase, K_m is 4.3×10^{-5}, and V_{max} is 34.5 γ P/mg. protein/10 min., while for ATP-ase K_m is 3.1×10^{-4}, and V_{max} is 70 γ P/mg. protein/10 min. (K_m for erythrocyte IPP-ase equals 5.4×10^{-4} according to Bloch-Frankenthal, 1954; and K_m for skeletal muscle ATP-ase equals 1×10^{-4} to 3×10^{-4}, according to Watanabe

et al., 1952.) Under these conditions, Q_p of the ATP-ase equals 300 as compared to 150 for the bull sperm ATP-ase (Nelson, 1954), and would probably be somewhat higher for purified sperm tail extracts. Further evidence substantiating the belief that two separate enzymes are involved derives from comparison of the effects of sulfhydryl reagents on the dephosphorylation of the two substrates.

Sulfhydryl inhibition of flagellar ATP-ase and IPP-ase. The different sulfhydryl inhibitors caused varying degrees of inhibition of the enzymes. ATP-ase is more sensitive than IPP-ase to the action of both N-ethyl maleimide and p-chloromercuribenzoate at an inhibitor concentration of 10^{-4} *M*, while sodium monoiodacetate inhibits both enzymes only very slightly (bar graphs, Fig. 6a). ($HgCl_2$ at the same concentration completely inhibits the ATP-ase.) P-chloromercuribenzoate, a mercaptide-forming agent, is more effective at higher concentrations, while N-ethyl maleimide, an alkylating agent, is relatively more potent at lower concentrations in inhibiting the sperm tail ATP-ase (Fig. 6b). This may reflect the fact that while N-ethyl maleimide is a specific sulfhydryl inhibitor, p-chloromercuribenzoate at the higher concentrations may be combining with other reactive and essential sites on the protein side-chains in addition to sulfhydryls (Boyer, 1959). Unfortunately, no attempt was made at the time to reactivate the enzymes by treatment with sulfhydryl compounds such as BAL (2, 3 dimercaptopropanol), glutathione or cysteine.

Discussion

The studies on the temperature dependence and the effects of sulfhydryl inhibitors are relatively straightforward. Kielley and Bradley (1956) reported that with calcium as activator, when approximately one-half of the sulfhydryl groups of myosin are titrated with either p-chloromercuribenzoate or N-ethyl maleimide a marked increase in ATP-ase activity occurs. Other distinguishing features of the myosin- and actomyosin-ATP-ase and sperm tail ATP-ase have already been considered (Nelson, 1955). Under the conditions of the present experiments, the flagella again differ from the muscle ATP-ases in exhibiting none of the sulfhydryl reagent activation; whether this is characteristic only of the purified enzyme or of Ca-activated ATP-ase, remains to be investigated.

Of particular interest has been the finding that the sperm tails actively dephosphorylate pyrophosphate. Mohri (1958) concludes that Hemicentrotus sperm tails which hydrolyze ATP are enzymatically inactive to a number of other phosphate esters among which he includes inorganic pyrophosphate. Heppel and Hilmoe (1951) describe an inorganic pyrophosphatase in bull seminal plasma, with a sharp optimum at pH 8.6. The bull seminal IPP-ase has nearly maximal activity in the absence of magnesium, while firefly (McElroy *et al.*, 1951, 1953) and yeast IPP-ase have an absolute requirement for Mg^{++}, as apparently does that of Mytilus sperm tail (although this requires further study). The bull seminal IPP-ase, unlike that of yeast and the sperm flagellum, shows no inhibition by increased substrate. Since metaphosphate, which also forms firm complexes with Mg^{++}, but is not acted on by the enzyme, inhibits yeast IPP-ase in the same concentration range as pyrophosphate, Heppel and Hilmoe (*loc. cit.*) attribute inhibition by high pyrophosphate to Mg^{++} binding. This interpretation is substantiated by the present studies involving the combined action of ATP and NaPP. Moreover, inorganic pyro-

phosphate inhibits magnesium-activated myofibrillar ATP-ase when the total concentration of ATP and NaPP exceeds that of the $MgCl_2$ (Perry and Grey, 1956), and decreases the light scattering of actomyosin solution in the presence of magnesium (Tonomura *et al.*, 1952) even though the pyrophosphate is not split. A number of the nucleoside triphosphates also possess this property of modifying, or interfering with the myosin or actomyosin interaction with ATP, and so the behavior of inorganic pyrophosphate is not unique in this respect (Hasselbach, 1956). However, when considered in conjunction with the activity of the enzyme inorganic pyrophosphatase, this substance assumes peculiar significance and invites speculation as to its possible role as a regulator of a specific cellular energetic reaction. To cite several instances, in addition to vertebrate muscle, for which inorganic pyrophosphate may also serve as an extractant, both the substrate and enzyme may be involved in such diverse activities as firefly luminescence and insect flight. McElroy and his co-workers (1953) report that the decrease in light intensity after mixing ATP, luciferin, luciferase, O_2, and Mg^{++} is due to the formation of an inactive complex of luciferase which depends on magnesium and a second protein, namely, IPP-ase. Addition of pyrophosphate causes a sharp increase in the light, but inhibitors of the pyrophosphatase (Mn^{++}, Ca^{++}, F^-) must be added to prevent the rapid decay of the high light intensity obtained with the pyrophosphate. (However, iodoacetate, even at concentrations of 10^{-3} and 10^{-2} *M* does not inhibit this pyrophosphatase.) Gilmour and Calaby (1953) suggest the possibility that pyrophosphate hydrolysis may have some importance as a source of energy for cellular processes, since locust thoracic muscle pyrophosphatase is three times higher than that of femoral muscle, and also refer to the report that the heat of hydrolysis of pyrophosphate is approximately 9000 cal./mole (Ohlmeyer and Shatas, 1952). It is unlikely that such an enzymatic reaction is without physiological consequence. An interpretation in harmony with the wide variety of evidences of pyrophosphate involvement in cellular processes may be deduced from evidence that pyrophosphate is one of the naturally occurring "relaxing" or plasticizing factors. Pyrophosphate duplicates the softening effect of ATP in glycerinated muscle fibers and sperm flagella, so-called "cell models" (Bishop, 1958a). Magnesium is essential for the production and maintenance of the extensibility and plasticity of glycerol-extracted muscle (Bozler, 1954a), by physiological concentrations both of ATP and of NaPP. Bozler (1954b) proposed that relaxation is caused by the inactivation of bound calcium and that the relaxed state is due to the formation of an enzymatically inactive protein-ATP-Mg complex. He suggests (p. 157) that "the effect of ATP depends on a balance between two antagonistic actions, contraction, which is caused by the breakdown of ATP, and a softening action like that caused by PP. Whether contraction or relaxation occurs then depends on which of these effects predominates."

["Elementary processes in muscle action," Morales *et al.* (1955) should be consulted for a review of the actions of two other naturally occurring modifying factors, myokinase and the system ATP-creatine-transphosphorylase + creatine phosphate, as well as EDTA, and the features held in common by these very different substances.] Bishop (1958b) believes that one or more of the relaxing systems may play roles in sperm model "motility," since glycerinated sperm twitch repetitively on addition of ATP, ADP, or ITP, while pyrophosphate increases the amplitude of the twitch induced by these substances in rodent sperm. However,

these models are capable of very little, if any, progressive movement, the rate of oscillation is usually slower than that of fresh sperm, the wave is not propagated, and the movements are occasionally restricted to one or another portion of the tail. In this connection, it is worth noting that inorganic pyrophosphatase is a water-soluble enzyme, and as such is one of several components extracted upon glycerination (Nelson, 1959). Isolated fresh sperm tails, also, may oscillate or twitch (Gray, 1958), and so the control or regulation of the undulatory flagellation is most likely an autonomous function of the flagellum itself. Initiation of motile activity may depend on "extraneous" excitatory factors, *e.g.*, the so-called dilution effect, hormones, partial pressure of O_2, or CO_2, etc. (*cf.* Mann, 1954). But once the sperm is activated, propagated contraction waves progress down each of the nine outer longitudinal fibers in sequence in such a fashion that while one fiber is contracting, the ones opposite are plastic, undergoing relaxation-activation cycles which immediately succeed their own contraction waves (Nelson, 1958b). This sequence could impart the spiral twist observed in the undulatory wave and perhaps as well the spiral thrust which characterizes the progressive movement of the sperm. In cytochemical terms this may be visualized as follows: The contractile protein-Mg-ATP complex is the condition of the "active" state. Upon contraction, a rigor-like state might ensue, except that pyrophosphate then combines with the contracted fiber through Mg^{++}, inducing the relaxation phase; but since NaPP cannot replace the contraction-inducing property of ATP, and the kinetics of dephosphorylation suggest that the two substances are mutually inhibitory at physiological levels, the NaPP "block" must be removed from the spatial proximity of the contractile site. This may be effected enzymatically by the Mg^{++}-activated IPP-ase. Now, locally resynthesized ATP may recombine with the protein in complex with Mg^{++} released from combination with pyrophosphate. This type of contraction-relaxation cycle, resembling a spatially compact reciprocating mechanism, obviates the necessity for invoking "long-range" migrations of reactants. The highly speculative nature of this scheme may eventually be resolved when the mechanism and site of resynthesis of the inorganic pyrophosphate are discovered, although this should not be an insurmountable objection to the working hypothesis, since both pyrophosphate and pyrophosphatase apparently occur in a wide variety of biological systems (*cf.* Bloch-Frankenthal, 1954).

Summary

1. Mytilus sperm tail ATP-ase temperature coefficient (Q_{10}) = 2; temperature optimum occurs in the range between 20° and 30° C.

2. The sperm tails actively dephosphorylate sodium pyrophosphate (NaPP) as well as ATP. Two separate enzymes are involved, which together with their substrates apparently compete for the magnesium ions in the medium.

3. $K_{M\text{-}ATP\text{-}ase} = 3.1 \times 10^{-4}$; $K_{M\text{-}IPP\text{-}ase} = 4.3 \times 10^{-5}$.

4. In low concentrations of ATP and NaPP, the amount of inorganic phosphate liberated is additive, while at higher concentrations, inhibition occurs.

5. ATP-ase is more sensitive than IPP-ase to sulfhydryl inhibition, although iodoacetate has only slight effect on both enzymes.

6. An hypothesis is proposed that regulation of the undulatory flagellar wave primarily resides within the flagellum itself, and is a function of the reciprocal

activity of two enzymes, ATP-ase in the contraction phase, and IPP-ase in the relaxation phase.

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VASCULAR BUDDING IN BOTRYLLOIDES[1, 2]

HIDEMITI OKA AND HIROSHI WATANABE

Zoological Institute, Tokyo Kyoiku University, Tokyo, Japan

In our previous paper (1957) it has been demonstrated that in *Botryllus primigenus*, in addition to the ordinary palleal (peribranchial) budding, new buds are formed also from aggregations of blood-cells at the base of ampullae, and the epithet "vascular" has been proposed for that kind of budding. The question at once arises whether this vascular budding occurs also in other members of the family Botryllidae. Our researches along this line have revealed that *Botrylloides violaceum* under certain circumstances propagates by vascular budding entirely analogous to that of *Botryllus*.

In this brief note the process of vascular budding in *Botrylloides* will be described and then compared with that of *Botryllus*.

Materials and Methods

The materials on which the following observations were made were living colonies of *Botrylloides violaceum* Oka, commonly found in the vicinity of Shimoda Marine Biological Station, Shimoda, Japan. As is well known, in *Botrylloides* the zooids are arranged in meandering systems instead of in circular systems as in *Botryllus*.

To facilitate observation, colonies were fixed on glass slides. The technique used for fixing the colonies, setting out the slides in the bay, etc. was essentially the same as that described in the paper of Oka and Usui (1944). Observations on living materials were supplemented, if necessary, with examination of sections.

We take this opportunity and express our thanks to the Director and staff of the Station for providing us facilities for carrying out this research. Thanks are also due to Miss Yoshiko Oshima for her helpful assistance in laboratory works.

Observations

Developmental cycle in the colony of Botrylloides

Developmental cycle in the colony of *Botrylloides* is exactly the same as in *Botryllus*. In both, the zooids in a colony are perfectly coordinated, so we can speak of the phases of a colony as a whole. A colony has four successive phases, which constitute a developmental cycle. For particulars see our previous paper (1957).

[1] Contributions from the Shimoda Marine Biological Station, No. 109.

[2] The cost of this research has been partly covered by the Scientific Research Expenditure of the Department of Education of Japan.

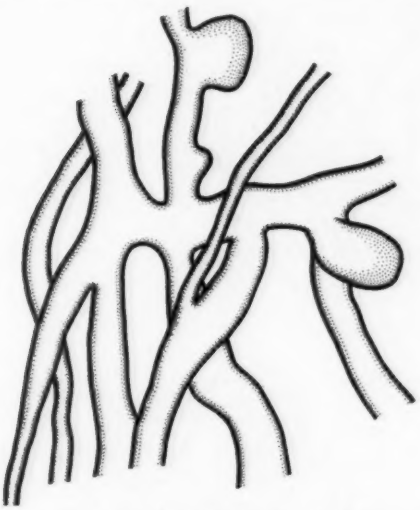

FIGURE 1. Test vessels immediately after isolation. × ca. 60

Conditions for the appearance of vascular buds

In the normal growth of the colony of *Botrylloides violaceum*, buds are formed exclusively from the palleal wall, *i.e.*, no sign of vascular budding is seen. When, however, a small piece containing ampullae and vessels but no zooids is cut out from the marginal part of a colony, vascular buds appear in it after 2 or 3 days.

In *Botrylloides* colonies, as in *Botryllus* colonies, numerous blood vessels traverse the test (Fig. 1) and terminate in contractile ampullae at the periphery of the

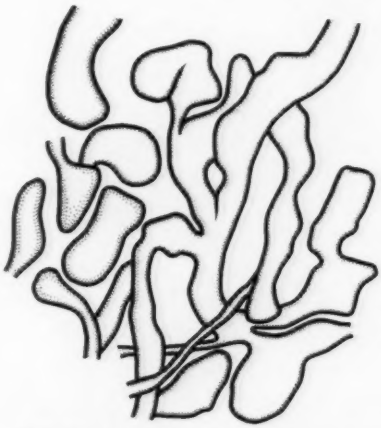

FIGURE 2. Test vessels in a one-day-old piece. × ca. 60

FIGURE 3. Vascular buds in a 3-day-old piece. × ca. 60

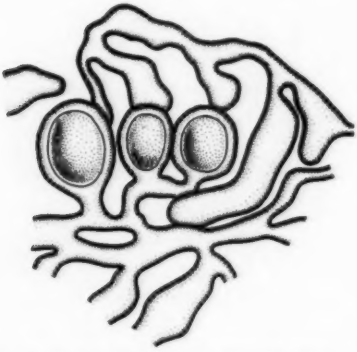

FIGURE 4. Vascular buds in a 4-day-old piece. × ca. 60

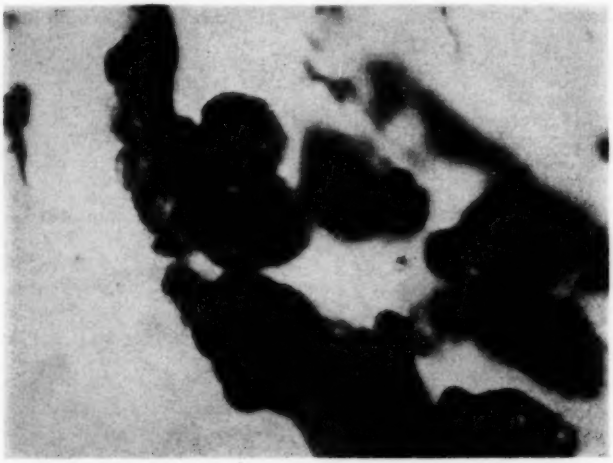

FIGURE 5. Section of a bud from a 2-day-old piece. × ca. 2000

colony. In a cut-out piece, the whole vascular system, inclusive of ampullae, strongly contracts. At the same time many club-shaped branches are sent out, and finally a dense network of anastomosing vessels is formed (Fig. 2). It is seen that a flow of blood is maintained in it. On such vessels the vascular buds are formed.

Formation of the vascular bud and its further development

It is seen in sections that the formation of the vascular bud is initiated by gathering of lymphocytes (diameter ca. 3–4 μ) under the epidermis of the blood vessel (Fig. 5). The initial number of lymphocytes is about 15–20 as in *Botryllus*. The development of a new zooid out of this cell mass follows exactly the same course as in *Botryllus*. At first, through intensive cell division a hollow blastula-like structure (diameter ca. 30–40 μ) is formed (Fig. 6); at the same time, the local epidermis of

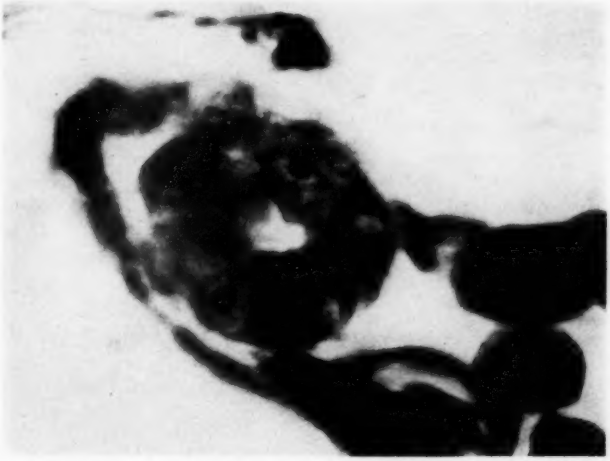

FIGURE 6. Section of a bud from a 3-day-old piece. × ca. 2000

the blood vessel gradually protrudes so as to wrap up the vesicle in itself (Fig. 3). The bud is now distinctly visible as such even in living materials. Morphologically, the vascular bud of this stage (diameter 50 μ) corresponds to the palleal bud of stage 3 except that it has no ova. Further development is the same as in the palleal bud. As an example, buds in a 4-day-old piece are shown in Figure 4.

Time of appearance

At whatever phase in the developmental cycle of the colony isolation may occur, vascular budding always begins two to three days after isolation and lasts about 24 hours. This means that vascular budding is not related to any definite phase of the original colony.

Vascular budding is strictly restricted to that period, for, as it seems, further bud-formation from the vascular wall is inhibited by growing vascular buds, and the

new zooids, once formed by vascular budding, propagate exclusively by palleal budding.

Site of appearance

At the time of budding, the vascular system in the isolated piece is represented by a network of blood vessels, the diameter of which varies from 45 μ to 120 μ. Unlike *Botryllus*, the vascular buds never appear at the bases of ampullae. Nor do they appear on the newly formed club-shaped endings. They are always formed along the walls of the old blood vessels.

Degeneration of the buds

At the end of the budding period, *i.e.*, 3 to 4 days after isolation, we could often count 60-70 newly formed buds, but not all of them continued to develop. As in the case of *Botryllus*, only those which surpass a certain size can develop and form perfect zooids, while the remaining ones undergo involution. An example of various sizes of buds is shown in Table I.

TABLE I

Various sizes of vascular buds in a piece 4 days after isolation

Diameter (in μ)	Number of buds
ca. 10	9
ca. 15	8
ca. 20	25
ca. 30	17
ca. 40	6
ca. 50	1
ca. 60	1
ca. 70	1
ca. 80	1
ca. 90	1
Total	70

Of these 70 buds, those larger than 40 μ continued to develop (11 buds, or 16%), while those under 30 μ soon began involution and finally disappeared without leaving any traces behind (59 buds, or 84%). In another case, of 35 buds once formed, only 6 (17%) developed into perfect zooids and formed a colony with two systems. Thereupon the colony began to grow by palleal budding.

DISCUSSION

Differences between Botryllus and Botrylloides

The formation of the bud itself is precisely alike in both forms. Yet, as to the conditions for and the time and site of the appearance of the vascular buds, there are differences between *Botryllus primigenus* and *Botrylloides violaceum* as stated below:

1. In *Botryllus*, vascular budding occurs in active colonies concomitantly with palleal budding. In *Botrylloides*, vascular budding is never seen under normal

conditions. Only when a small piece of a colony devoid of zooids is isolated, vascular budding is, so to speak, evoked in it.

2. In *Botryllus* the appearance of the vascular buds is limited to a certain phase (late phase B) in the developmental cycle of the colony. In *Botrylloides* vascular buds can be formed at any phase of the original colony.

3. In *Botryllus* the buds are located strictly at the bases of the ampullae, while in *Botrylloides* the buds are scattered across the colony along the walls of the vascular system.

Actually all these differences are the same as existing between *Botryllus primigenus* and *Botrylloides gascoi* except on one point. In *Botrylloides violaceum* we could repeatedly observe vascular budding in isolated pieces. In *Botrylloides gascoi*, however, an isolated piece devoid of zooids never regenerated a colony, and none of the ampullae in such a piece showed the least tendency towards budding (Bancroft, 1903, p. 451). Probably this led Bancroft to suppose that the presumable vascular buds observed by him in an aestivating colony were developed not from vessels but from the zooids of the original colony before these had degenerated entirely and later severed their connections with the parent zooids. It is to be hoped that some future investigator will repeat the experiments with *Botrylloides gascoi*.

Regulation acting upon the vascular buds

Botryllids are known for their zooids being most perfectly coordinated.

In *Botryllus*, the vascular buds are formed a little later than the corresponding palleal buds, but they are soon synchronized with these. Buds formed too late are forced to degenerate, thus being eliminated from the colony. All this regulating influence is supposed to come from the pre-existing active zooids.

In *Botrylloides*, the vascular buds are formed in the absence of any pre-existing zooids. Yet, sooner or later, all the newly formed zooids are synchronized, and, as in *Botryllus*, buds formed too late are eliminated from the colony. Possibly with the growth of early buds a new coordinating system is established in the piece and this regulates the growth of late-coming buds.

Budding in Botryllidae

Now that vascular budding has been demonstrated also in *Botrylloides*, it is to be assumed that this kind of budding is rather widely distributed among the family Botryllidae.

It is generally believed that stolonial budding—of which vascular budding is only a special form—is a rather primitive type of budding, while palleal budding is phylogenetically a relatively late acquisition. Moreover, palleal budding is so unique in nature that it cannot be derived from any other known kind of budding. So it has been assumed "that the primitive pleurogonid, undoubtedly derived from an enterogonid, had already lost any such capacity for budding, and that within the new order the Botryllinae have re-acquired it by a new method" (Berrill, 1950, pp. 50–51). That the original capacity for budding has not been completely lost in botryllids is clear from our investigations on *Botryllus* and *Botrylloides*. Only, with the rise of the new method of palleal budding, it has been more and more suppressed. In *Botryllus primigenus* vascular budding still takes part, though concomitantly with palleal budding, in the natural growth of the colony. In

Botrylloides violaceum, and probably *Botrylloides gascoi*, vascular budding is totally suppressed in the ordinary life of the colony. Only in the absence of zooids the otherwise latent capacity of forming buds from the walls of the blood vessels is called forth as a means to save the colony from extinction.

Summary

1. Generally *Botrylloides violaceum* Oka propagates by palleal budding alone. Only when a small piece of a colony devoid of zooids is isolated, new buds are formed from the walls of the test vessels, *i.e.*, vascular budding appears.

2. As in *Botryllus*, these new buds are formed from aggregations of lymphocytes under the wall of the test vessels.

3. Unlike *Botryllus*, the buds are not bound to any definite sites, but are distributed irregularly along the walls of the vascular system.

4. The buds generally appear 2–3 days after isolation, at whatever phase of the original colony the isolation may occur.

5. Major difference between *Botryllus* and *Botrylloides* is that in the former vascular budding coexists with palleal budding, while in the latter vascular budding is totally suppressed in the normal life of the colony.

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AXENIC CULTIVATION OF THE BRINE SHRIMP ARTEMIA SALINA

LUIGI PROVASOLI AND KAGEHIDE SHIRAISHI

Haskins Laboratories, New York 17, N. Y., and Dept. of Fisheries, Faculty of Agriculture, Tohoku University, Sendai, Japan

In a previous paper (Provasoli, Shiraishi and Lance, 1959) we have added to Gibor's (1956) observations that related species of algal flagellates may be either good or bad food for *Artemia*. This idiosyncrasy may depend upon nutritional deficiencies in the algal food, on toxic metabolic products, or even upon some nutrient in excess. One way to attack this ecological problem is to grow *Artemia* on a non-living medium as a step toward a chemically defined medium and, finally, identification of all its nutritional requirements.

The present paper concerns the first stage, *i.e.*, the growth of *Artemia* on a non-living complex medium.

Materials and Methods

The amphigonic American race of *Artemia salina* was employed. Utah brine shrimp eggs (Aquarium Stock Co. Inc., 31 Warren Street, New York 7, New York) proved more satisfactory than other samples tried in respect to percentage of hatching and speed of development.

Sterilization of the eggs. Durable eggs of *Artemia* obtained commercially always contain many dead dried eggs whose chorion is cracked. These eggs are lighter and cannot be disinfected as rapidly as the intact viable ones and should be eliminated at the onset to avoid infections from the inoculum. The technique of disinfection is a modification of the one employed by Gibor (1956).

The dead eggs, being lighter than the viable ones, are eliminated by the flotations in sea water. The eggs are disinfected in screw cap tubes for 10 minutes in Merthiolate solution (1 : 1000 in H_2O) + 0.2 ml.% of a 10% solution of Aerosol OT. to improve wettability. The disinfectant is decanted and the eggs washed in three baths of sterile sea water. The egg slurry is distributed into several tubes of a sterility-test medium (*STP*, Table I) and allowed to hatch 2–3 days at 22–26° C.). Contamination generally shows in 2–4 days. The new-born nauplii develop to second metanauplii at the expense of the reserves of yolk within 4–7 days; the third metanauplii, if not fed, die.

The metanauplii are transferred into a nutrient medium 1–2 days after the first nauplii have hatched, to secure a more uniform inoculum in respect to age; hatching is spread over several days.

The growing larvae consume the particulate food rapidly and must be transferred approximately every 6–8 days, especially after the fourth stage. Transfers are made with Pasteur pipettes connected to a mouthpiece by rubber tubing, to allow a clear view while fishing the larval forms. The later larval stages and the young adults defy suction unless sucked head first, while swimming toward the tip of the pipette. To avoid air-borne infection we used a transfer hood (top and back glass,

TABLE I

STP medium

Sea water	80 ml.
H_2O	15 ml.
Soil extract	5 ml.
$NaNO_3$	20 mg.
K_2HPO_4	1 mg.
Na H glutamate	50 mg.
Glycine	10 mg.
DL-Alanine	10 mg.
Vitamin No. 8A*	0.1 ml.
Trypticase (B.B.L.)	20 mg.
Yeast autolysate (Albimi)	20 mg.
Sucrose	0.1 g.
pH 7.5	

* See Table 4, p. 408 in Provasoli *et al.* (1957).

open in front) with a "Letheray" germicidal UV lamp (see description and figure in Provasoli, Shiraishi and Lance, 1959).

Preparation of media. The following medium (Table II) allows growth to adulthood.

TABLE II

Complete medium

STP (1)	100 ml.
Cholesterol (2)	200 μg.
Dehydrated liver infusion No. L 25 (3)	100 mg.
Trypticase (B.B.L.)	300 mg.
Alkaline-hydrolysed nucleic acid (4)	40 mg.
Acid-hydrolysed DNA (5)	10 mg.
Sucrose	200 mg.
Vitamins mix Art. II (6)	1 ml.
Paramecium factor (7)	5 mg.
Glutathione (8)	30 mg.
Ascorbic acid (8)	3 mg.
Horse serum (aseptic)	5 ml.
Rice starch (9)	500 mg.
pH 7.5	

(1) See Table I.
(2) Dissolved in ethanol.
(3) Oxoid Ltd., England.
(4) Yeast RNA brought to pH 9.0 with NaOH and steamed for one hour.
(5) Herring DNA brought to pH 1.5–2.5 with H_2SO_4 and steamed for two hours.
(6) Vitamins mix Art. II

Thiamine HCl	10 mg. %
Biotin	0.5 mg. %
Folic acid	7 mg. %
Nicotinic acid	50 mg. %
Choline	500 mg. %
Ca pantothenate	70 mg. %
Pyridoxine HCl	8 mg. %
Carnitine	20 mg. %
Riboflavin	0.1 mg. %

(7) Paramecium factor was kindly supplied by Dr. D. M. Lilly (1 part dried yeast cells + 1 part H_2O, autolyzed at 58–60° C. for two hours; the particles are centrifuged and the supernatant vacuum-dried; to obtain a fairly good suspension, bring to a boil.
(8) Fresh solutions of glutathione and ascorbic acid, sterilized by glass-filtration, are added before inoculation.

At first the starch was added to autoclaved media as a slurry. Rice-starch powder is mixed with glass beads (200 μ diameter-Superbrite type 100, Minnesota Mining and Manuf. Co.) and sterilized for two hours at 180° C.; sterile water is added to form a slurry.

We found later that *Artemia* ingests cooked starch equally well; this eliminated one aseptic addition. To prevent the starch from forming a semi-solid mass during sterilization, 500 mg.% of starch powder is added to the complete medium (minus other aseptic additions) before sterilization; the medium is brought to a boil while being stirred vigorously with a glass rod or on a heating plate equipped with a magnetic stirrer. The starch, on boiling, forms small floccules which become larger upon cooling but remain acceptable to *Artemia*. The medium is then tubed and autoclaved.

Miscellaneous preparations:

a) The cholesterol is generally added as an alcohol solution. It has also been employed absorbed on cellulose powder ("Celluflour," Turtox) following the techniques of Singh and Brown (1957) (1 mg. of cholesterol dissolved in 10 ml. ether is mixed with 200 mg. Celluflour; let dry, then added to media before autoclaving).

b) Fatty acids absorbed on starch: 980 mg. of the following fatty acid mixture (proportions of House and Barlow, 1956) palmitic acid 200 mg. + stearic acid 100 mg. + oleic acid 480 mg. + linoleic acid 150 mg. + linolenic 50 mg. are dissolved in ether and mixed with 10 g. of starch; after evaporation of the ether, the powder is sterilized in ethylene oxide for twelve hours; the powder is made aseptically into a slurry with water, and a solution of NaOH or $Ca(OH)_2$ added to neutralize the fatty acids.

c) Albumen (bovine) fraction V (N.B.C.) is dissolved in sea water, glass-filter-sterilized, and added aseptically.

Fraction V was employed also as a carrier of cholesterol and the fatty acids mixture. Sterile solutions of fraction V and cholesterol (dissolved in ethanol) are mixed and added after autoclaving. Two ml. of a sterile 8% solution of Fraction V were mixed aseptically with 2 ml. of an autoclaved fatty acid mixture (dissolve in 50 ml. of H_2O, 1 ml. of concentrated NaOH, stearic acid 24 mg., palmitic acid 54 mg., linoleic acid 36 mg., linolenic acid 12 mg., oleic acid 0.12 ml., adjust to pH 8.0 with HCl, autoclave).

Interpretation of Results

To compare the nutritive value of different foods and supplements we needed to recognize the various larval forms by characters easily visible by inspection of the tubes with a hand lens. We could not resort to the fine external morphological

characters employed by Heath (1924) to divide in 13–15 instars the development of *Artemia* from egg to adult; these differences can be evaluated only with a microscope. We decided therefore to employ, slightly modified, the arbitrary nomenclature of Barigozzi (1939) which divides the life-cycle in stages of development clearly distinguishable with the hand lens.

The nauplius is small, roundish, yellow-pink (first instar of Heath). Metanauplius I is small, triangular, yellowish (second instar of Heath). Metanauplius II is similar but bigger (third instar of Heath).

Metanauplius III. "Small" III T-shaped, longer, thin, shows a visible segmentation in the upper thoracic region (fourth instar of Heath); "big" III (1.5 mm.); the first 3–5 thoracic limbs are well developed but do not move well (fifth instar of Heath). The first three metanauplii stages are easily distinguished from the other stages, even with the naked eye, by their jerky swimming: at these stages propulsion depends upon the characteristic backward and forward paddle-like movement of the long second antennae.

Metanauplius IV. "Small" IV (2 mm.). The first thoracic limbs are now moving well and the movement of *Artemia* is a combination of jerks and swimming in circles (sixth instar of Heath); "medium" IV (2.5 mm.) swims on its back gracefully in circles; most of the thoracic limbs are fully articulated and move rhythmically like rippling waves (seventh instar of Heath); "big" IV (3.5 mm.) bigger in size, abdomen longer and slender, the second antennae smaller than the limbs and lying parallel to the head (eighth instar of Heath).

"Juveniles" (5–7 mm.) have stalked eyes, the abdomen elongates and becomes segmented, at the tip of the abdomen the furca becomes evident; they have a slender appearance and resemble adults but are much shorter (ninth-eleventh instars of Heath).

Adults (7–10 mm.). The males have long claspers (modified overgrown second antennae); the females have a slender head and a conspicuous egg-pouch right below the last pair of limbs (twelfth-fifteenth instars of Heath).

We record twice a week the stage reached in each tube. As in many insects, some phases of the life-cycle of *Artemia* seem more critical than others: the transition from the third to the fourth stage and the one between "big IV" to "juveniles." In general, a good way to evaluate the effect of the different supplements is to compare (a) the days required to reach the "small IV" metanauplius, (b) the days needed to reach adulthood, and, if they do not become adults, (c) the stage reached at death and days elapsed since birth.

We generally inoculate 5–8 larvae per tube. Not all develop into adults even in the best media although in these media most do. Quite often, especially from the III metanauplius onward, "black disease" develops: black spots, consisting of fine melanin granules, develop at the lobes of the phyllopodia, especially on the dactylopodite. The incidence of black-spotted individuals is sporadic and could not be correlated with any particular nutritional deficiency; it might be simply a difficulty in molting, *i.e.*, left-over parts of the previous cuticle may impede normal development. We cannot say how much these spots affect normal growth and if they are harmful; in complete media (Table II) the adults often had black spots from the IV metanauplius on, yet they could become adults. Black disease is often common; until its causes and effect on the health of the larvae are known it

is impossible to use accurately the percentage survival of a mixed population of normal and black-spotted larvae as an index of the nutritional status.

Results

a) *Particulates*

Artemia is a voracious particle-feeder as we amply observed when rearing them on living flagellates. We thought that this behavior could be exploited to increase the ratio of ingestion (drinking) of the nutrients added as solutes, because little absorption can be expected by an arthropod except from the middle intestine, the rest of the body being clad in chitin. An ideal situation would be to have a nutritionally inert attractive particle and to supply nutrients as solutes, thus permitting the application of the usual microbiological techniques for replacing complex organic substances with chemically defined components. We tried a variety of particles, many of them nutritionally rich, because we did not know whether the organisms could withstand a medium rich enough in solutes to support their growth. Early experiments had shown that 0.6% Trypticase inhibits *Artemia* and that *Tigriopus* is even more sensitive to organic solutes. The following compounds were ground fine (between 5–20 μ) in a colloidal mill, sterilized by dry heat, and added aseptically as water suspensions to the liquid part of the medium. The liquid (STP, Table I, + a vitamin mix, and 100 mg.% of "Oxoid" liver infusion, no. L 25) employed at the time is nutritionally deficient and, in the absence of particles, *Artemia* grow only up to medium-sized III metanauplii. Additions of 150 mg.% of particulate blood fibrin, yeast cells, corn protein, lactalbumen, Cerophyll, casein, Celluflour, and rice polishings were ineffective, while fish meal or gluten permitted reaching the IV in 23–25 days; rice starch did so in 35–40 days. Rice starch was selected because it is, if digested, mainly a carbon source. It is almost devoid of impurities of other important nutrilites (as is gluten) and therefore offers the possibility of defining requirements for amino acids, proteins, fats, and vitamins.

Larval forms of *Artemia* are voracious: suspended particles are quickly transformed into fecal pellets. We therefore raised the particulate starch to 0.5% and kept it suspended as much as possible by shaking and homogenizing the medium twice daily. Later on, when solutions allowed growth beyond the third metanauplius, we had to transfer the growing metanauplii every 7 days to a fresh medium and to increase the volume of the medium from 5 ml. to 10 ml. Five ml. of medium in 20 × 125 mm. tubes are better for the growth of young larvae (up to the early stages of "small IV") because such larvae swim poorly, feed mostly at the bottom, and need a medium well aerated by a large surface exposed to the air. Later stages are continuously swimming and stirring the medium. Perhaps the later larval stages would grow faster if the media were changed even more often, but this requires much patience and increases contamination (see "Methods"). It was found later that the starch can be added before sterilization if it is precooked while stirring the medium (see "Preparation of Media"); the resulting floccules are still ingested, and remain more easily in suspension.

When we found a medium allowing growth to adulthood (Table II), we reinvestigated the necessity of particles. The liquid part of the medium (excepting the heat-sensitive components of the medium) was autoclaved, filtered through

paper, then glass-filter-sterilized, and dispensed aseptically into tubes. Sterile solutions of glutathione, ascorbic acid, and the serum were added last.

This medium is clear and devoid of visible particles. The nauplii of *Artemia* in this medium reached at best the stage of "big III" metanauplii. In the same batch of medium to which was added aseptically a sterile starch slurry, *Artemia* reached adulthood. A similar experiment was done recently but with another medium allowing growth to adulthood; again the absence of particles prevented growth beyond the third metanauplius.

b) *Solutes*

Trypticase and nucleic acids. In preliminary experiments it was soon discovered that addition of Trypticase (0.3%) and nucleic acids to the liver extract speeded growth greatly; the IV metanauplius stage was reached in 12–19 days but growth stopped at medium-size metanauplii. Whole blood (1 ml./100) and a suspension of red blood cells, as substitutes for the starch particles, did not speed growth or allow a more advanced stage; yeast cells (autoclaved) were inhibitory.

Vitamins. Since the level of the vitamin mixture initially used (Table I) was far below the levels for insects, we suspected that the medium was mainly deficient in vitamins. Tentatively, we chose concentrations and ratios similar to those employed for insects, but at the lower limits because in earlier experiments we found that cholesterol was already inhibitory at values which are low for many insects. Biotin, pyridoxine, folic acid, nicotinic acid, and choline, added singly and in combinations, either affected general vitality (*i.e.*, vigorous swimming), speeded the time required for reaching the fourth metanauplius stage, or permitted growth up to "very big" IV metanauplii. Therefore we designed richer and more complete vitamin mixtures (see latest in Table II). To see whether some vitamins were present in suboptimal or toxic concentrations, we removed singly each vitamin and added it at different concentrations. Thiamine and folic acid proved limiting, indicating that Trypticase, liver extract, and serum at the levels employed in the complete medium (Table II) are inadequate sources of these vitamins for *Artemia*. Adulthood was reached in the complete medium and no inhibitions were found up to the following maximal concentrations tried (wt./100 ml. final medium): thiamine 200 μg., biotin 30 μg., folic acid 300 μg., nicotinic acid 1 mg., pantothenic acid 3 mg. Choline did become inhibitory between 3 and 10 mg., pyridoxine between 50 and 100 μg.%, and riboflavin between 0.1 and 1.0 mg.%.

Serum, glutathione, and paramecium factor. Adults were not obtained until horse serum, glutathione, and paramecium factor were added to the medium. Serum alone reduced the time to reach the IV metanauplius from 29 days (no serum) to 19 days (2 ml./100), and to 13 days (4–10 ml./100); increasing the serum allowed growth up to "big" IV metanauplii.

In the absence of serum, 20–40 mg.% of glutathione enabled the metanauplii to reach the IV stage in 16 days and become "small" IV. Paramecium factor alone or in combination with glutathione seems ineffective, but when added to the combination glutathione plus serum, allows adulthood. Under our conditions, only "very big" IV or juveniles were produced by serum + glutathione; this combination was quite effective in speeding growth; the IV metanauplius was reached in 13 days. For production of adults the serum should reach the level of 3 ml./100

or more (up to 10 ml./100); when the serum was below 3 ml./100, depending on the quantities of serum added, only "big," "medium," or "small" IV metanauplii were produced.

Cysteine can substitute for glutathione in eliciting adulthood: 10 mg.% cysteine was as effective as 20–30 mg.% glutathione.

Horse serum can be substituted with a filter-sterilized solution of dried beef serum (Difco).

The active factors present in serum are heat-resistant: both supernatants of the autoclaved horse or Difco beef serum, glass-filter-sterilized and added aseptically, are as effective as serum.

Cholesterol and fatty acids. Some attempts were made to replace serum. Five milliliters of serum supply, *inter alia*, much neutral fat, lecithin, cephalin, and cholesterol. The medium without serum, except for the concentrations brought in possibly by 100 mg. of Oxoid liver, has no fatty acids and only 200 μg.% cholesterol. Some insects require long-chain unsaturated fatty acids (linoleic acid, linolenic acid); all require cholesterol. Early experiments had shown that cholesterol above 500 μg.% becomes inhibitory, but these experiments were done with poor media. In comparable media, linoleic acid was indifferent at 1 mg.% and inhibitory at 10 mg.%. With better media, cholesterol absorbed on Celluflour inhibited at or above 6 mg.% and linolenic acid was inhibitory above 1 mg.% and became rapidly toxic. Neither cholesterol or linolenic acid replaced the serum. The fatty-acid mixture absorbed onto starch powder was indifferent at 2–5 mg.% and quite toxic above. Thinking that the toxicity might be caused by the acidity of the acids, the sterile fatty-acid starch slurry was adjusted with NaOH or $Ca(OH)_2$; the Na salts are far more toxic than the Ca salts.

Cholesterol, or the fatty acid mixed with serum albumen fraction V, also did not replace serum. However, 200 mg.% of fraction V alone allowed normal adults in two months instead of 25 days; growth up to the IV metanauplius was as in serum. Soya lecithin became rapidly toxic; "Gliddex I" (a refined "lecithin" containing 4% lecithin, 29% cephalin, 55% inositol phosphatides, and 4% soybean oil) absorbed either on starch or casein, is well tolerated up to 10–15 mg.%. However neither lecithin, Gliddex I or other "lecithins" replaced serum.

Discussion

While *Artemia* can be grown without living food and tolerates concentrations of solutes sufficient to permit development to adults, it does not grow wholly on solutes. The particles are needed to stimulate filter-feeding, thus allowing ingestion of enough nutrient solutes for a normal growth rate.

The studies of Croghan (1958a, 1958b) on osmotic regulation in *Artemia* show: *a*) that the cuticle of the branchiae (metepipodites) of the first 10 pairs of the phyllopods is the only part of the external body cuticle that is appreciably permeable in the adults; in the young larvae, where the phyllopods have not yet developed, the neck organ is the permeable region; *b*) the branchiae are the site of active NaCl excretion; *c*) the gut epithelium controls internal water balance by water uptake; *d*) *Artemia* continues to swallow the medium even when devoid of particles. The swallowing behavior of the adults was indicated by the red coloration of the gut walls a few hours after *Artemia* were put into a filtered phenol red solution.

Our experiments show that the rate of swallowing of a particle-free medium is probably very low in the early metanauplii stages—certainly insufficient to provide enough nutrient solutes for growth. Particles stimulate swallowing. Since all the nutrients in our media are in solution, Trager's (1936) conclusion, based on *Aedes aegypti*, that solutes are utilized for growth, applies to Crustacea. Although other invertebrates, like some ciliates, can be grown on solutes, perhaps some phagotrophs, including Crustacea, living in oligotrophic waters, may be obligate or partial phagotrophs because particulate feeding, besides increasing the ingestion of nutrient solutes, does not affect their inability to withstand concentrations of organic solutes high enough to support growth (Provasoli, 1956).

The medium allowing growth to adults (Table II) is still quite complex; it offers, however, more possibilities of dissection than the only other known axenic medium for a crustacean—the blood-glucose mixture of Treillard (1924) for *Daphnia*. It was exciting to have started with an inadequate medium because each experiment permitted the demonstration of some nutritional needs, some of them reflecting obvious requirements, as was the effect of Trypticase and nucleic acids. It is remarkable that thiamine and folic acid were required even in the presence of Trypticase and liver extract which are ordinarily adequate sources of these vitamins. Glutathione is also required and was replaceable by cysteine. So far only another arthropod, the mosquito *Aedes aegyptii*, requires glutathione (Singh and Brown, 1957), even in the presence of adequate cysteine. Interestingly, the "feeding reaction" of *Hydra* is controlled by glutathione which acts as a specific "feeding hormone"—it is not replaceable by cysteine, ascorbic acid or other donors of SH groups (Loomis, 1955).

Serum is required for the full development of adults. The active components of serum are heat-stable. We could not replace serum with mixtures of fats and cholesterol. These results are only indicative: lack of effect may be due to toxicities of some components of the fatty acid mixture or failure to avoid toxicity by presenting them to *Artemia* on the proper fat carrier.

However, if the toxicity of fatty acids is the cause, it might explain some of the nutritional idiosyncrasies found previously (Provasoli, Shiraishi and Lance, 1959). Utilization of flagellates as food may depend as much upon their providing *Artemia* with all the nutrilites needed as with their lacking toxic substances and vice versa when they are not utilized as food.

For *Artemia* we found that especially in the *Chlorophyta* several species, even strains, were inadequate as food while others were not. Chlorophytes are known to produce toxic unsaturated fatty acids such as chlorellin (Spoehr *et al.*, 1949). Indeed this might well be a characteristic of the *Chlorophyta*. Proctor (1957) found that *Haematococcus* is particularly sensitive to palmitic, oleic, and linoleic acids, and also to substances produced by cultures of *Chlamydomonas reinhardii*. The substances produced by *Chlamydomonas* are steam-distillable and fat-like, quite probably a mixture of unsaturated fatty acids. The accumulation of oil droplets is readily observed in different species of *Polytoma*, the colorless counterpart of *Chlamydomonas*. During the logarithmic phase the cells are full of paramylum granules, but as they pass the peak of growth the starch is replaced in great part by fat droplets. This might explain the toxicity of aged cultures of *Chlorella* to *Daphnia magna* found by Ryther (1954).

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SUMMARY

1. *Artemia salina* can be grown aseptically to adults in a non-living medium.

2. The components of the medium are: sea water, Trypticase, liver infusion, hydrolysed RNA and DNA, serum, sucrose, cholesterol, paramecium factor, glutathione, a mixture of B vitamins, and starch particles.

3. Glutathione, thiamine, and folic acid were found essential even in the presence of Trypticase and serum. Glutathione can be replaced by cysteine. Horse or beef serum (Difco) supply unidentified heat-stable nutrients. Cholesterol and mixtures of fatty acids become rapidly toxic, and do not replace serum.

4. *Artemia* is a voracious particle feeder and transforms the starch particles rapidly into fecal pellets. In the absence of starch particles, the liquid part of the medium, though containing all the nutrients, supports growth only to the third-stage metanauplii. This indicates that the rate of ingestion (swallowing) of liquids is too low to support continuous growth and that the particles are necessary to increase the swallowing reaction.

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LARVAL DEVELOPMENT OF THE SAND CRAB EMERITA TALPOIDA (SAY) IN THE LABORATORY[1,2]

GEORGE H. REES

Duke University Marine Laboratory, Beaufort, N. C.

Present knowledge of the larval development of *Emerita talpoida* (Say) is limited to two early reports, both under the generic name *Hippa*. Smith (1877) described three zoeal stages, which he called second, third, and last zoea, and a megalops stage, all from the plankton of Vineyard Sound, Mass. Smith was unable to obtain a first zoea, as females brought into the laboratory invariably cast off their eggs before they hatched. Faxon (1879a) was able to obtain the first zoea from eggs hatched in the laboratory. He was unable to rear any larvae through the first molt into the second stage, but ventured the opinion that one or more stages remained to be discovered between the first and the earliest described by Smith.

The larvae of two other species of *Emerita* have been investigated, *Emerita asiatica* by Menon (1933), and *Emerita analoga* by Johnson and Lewis (1942). Johnson and Lewis were able to obtain the first zoea from eggs hatched in the laboratory, but were unable to maintain the larvae through the first molt. One individual did enter the second stage after 34 days, but died soon afterwards. On the basis of the first zoea obtained in the laboratory and other stages from the plankton, Johnson and Lewis describe five zoeal stages. In addition, they state that a number of specimens were collected which appeared to be intermediate between Stage III and Stage IV, and which they called, for convenience, "Lower Stage IV." Menon (1933) lists five zoeal stages for *Emerita asiatica*, all of which were obtained from the plankton.

The present paper is a description of the larval development of *Emerita talpoida* (Say) based on observations of larvae reared in the laboratory.

The author wishes to express his sincere appreciation to Professor C. G. Bookhout, under whose guidance and direction this work was done and who read and suggested improvements in this manuscript. Thanks are also due to Dr. John D. Costlow, Jr., for his many helpful suggestions during the course of the experiment.

Methods

Ovigerous females of *Emerita talpoida* were collected on the beach at Fort Macon, N. C., and held in the laboratory in large fingerbowls until hatching occurred. If unmolested, the females remained quiet in the fingerbowls until the time of hatching. At this time they became active and swam in short spurts around the sides of the container. At each spurt of swimming activity a cloud of

[1] Part of a thesis submitted to the graduate faculty of Duke University in partial fulfillment of the requirements for the degree of Master of Arts.

[2] Present address: Radiobiological Investigations, U. S. Fish and Wildlife Service, Beaufort, North Carolina.

larvae was released. The larvae began actively swimming immediately upon hatching, with no intervening prezoeal stage.

Groups of ten newly hatched larvae were placed in four-inch fingerbowls of sea water which had been filtered through glass wool and inoculated with 200,000 units of penicillin per liter. There were ten such bowls. Other larvae were reared in mass cultures in fingerbowls containing approximately 200 individuals each. Each day the larvae were transferred by means of a pipette to clean bowls of filtered, inoculated sea water. To each bowl each day was added a quantity of *Nitzschia* sp. and newly hatched *Artemia* nauplii. The exception to this was one of the mass culture bowls, to which only *Nitzschia* sp. was added. The zoea were observed to capture and feed upon the *Artemia* nauplii quite readily.

The fingerbowls which originally contained ten larvae each were examined daily and a record made of the number of individuals surviving and the number in each stage of development each day, based upon exuviae found. Larvae in each stage were removed from the mass culture dishes and preserved in 70 per cent alcohol.

Throughout the experiment the larvae were maintained at a temperature of 30.0° C. and under constant illumination from daylight fluorescent lights. The sea water in which the larvae were reared varied in salinity from 28.2 parts per thousand to 35.1 p.p.t., with a mean of 32.2 p.p.t.

Results

In the mass culture dish to which only *Nitzschia* sp. was added all individuals died while still in the first zoeal stage. In the other bowls, to which newly hatched *Artemia* nauplii were added in addition to *Nitzschia*, some of the individuals eventually entered the megalops stage after passing through six or seven zoeal stages. The majority of the individuals which became megalops did so after passing through six distinct zoeal stages; a few individuals went through an additional molt between the sixth zoeal stage and the megalops. The only morphological difference apparent between the sixth zoeal stage and the seventh zoeal stage was an increase of one or two setae on the exopods of the maxillipeds. The appearance of a seventh zoeal stage in a few individuals does indicate, however, that the number of molts through which an individual passes during larval development is not fixed and/or inflexible.

The number of individuals which entered each stage and the average duration of each stage are given in Table I. Although a few deaths occurred during the intermolt period, most of the individuals which died did so at the time of molting. The difficulty in molting was usually the result of the old exuvia adhering to the new exuvia, generally on the maxillipeds and near the tip of the rostral spine. This failure of the old exuvia to detach from the new may be due to a physiological weakness existing in some of the zoea. Whether this weakness also exists in nature is a matter for speculation.

The shortest length of time that it took any individual to become a megalops was 23 days, the longest was 33 days. The average length of the pelagic larval life in the laboratory was 28 days.

With each zoeal molt the number of setae on the exopods of the first and second maxillipeds increased by either one or two. This change in the number of setae

TABLE I

Number of individuals out of the original 100 which entered each stage of development and the average duration of each stage

Stage	I	II	III	IV	V	VI	VII	Megalops
Number of individuals	100	94	75	71	63	45	5	15
Average number of days spent in stage	3	4	3	4	5	9	3	

on the maxillipeds was found to be an accurate indication of the number of molts through which an individual reared in the laboratory had passed.

DESCRIPTION OF THE LARVAE OF E. TALPOIDA

FIRST ZOEA (FIG. 1)

The first zoeal stage is similar to the first stage of *Emerita asiatica*, as described by Menon (1933), and *E. analoga*, as given by Johnson and Lewis (1942). The smoothly rounded carapace is translucent, colorless, and without the lateral spines which are characteristic of subsequent stages. The rostrum is short and broad. The eyes are stalked. The eyestalks are short and thick and lie close against the carapace, directed somewhat posteriorly. The abdomen projects almost straight downward from the carapace and is flexed so that the telson is carried beneath and nearly parallel to the carapace. The exopods of the maxillipeds bear four plumose setae.

Antennules (Fig. 8). These short, unjointed appendages are thick at the base and taper to a blunt point where three setae of about equal length are borne. These setae are slightly longer than the body of the appendage.

Antennae (Fig. 14). The antennae at this stage are rather stubby appendages, produced on the outer side into a spine-like process. From the base of the outer spine there arises a somewhat slenderer dentiform process of about the same length. At the base of this inner process there is a much smaller spine. The form of the antennae is relatively unchanged through the first four zoeal stages, the first indication of a flagellum not appearing until the fifth zoeal stage.

Mandibles (Fig. 20). The mandibles grow out ventrally and then make a

TABLE II

Relative size of larvae in each stage reared in the laboratory. Based on average measurements of 10 or more specimens. Dimensions are given in mm.

Stage	I	II	III	IV	V	VI	Megalops
Max. length of carapace	0.57	0.68	0.88	1.13	1.60	1.90	2.30
Max. width of carapace	0.46	0.56	0.67	0.84	1.00	1.30	1.80
Length of abd. plus telson	0.80	0.84	1.30	1.50	1.80	2.40	1.90
Length of rostrum	0.20	0.68	1.27	1.55	2.40	2.90	—
Length of lateral spine	—	0.30	0.44	0.60	0.80	1.00	—

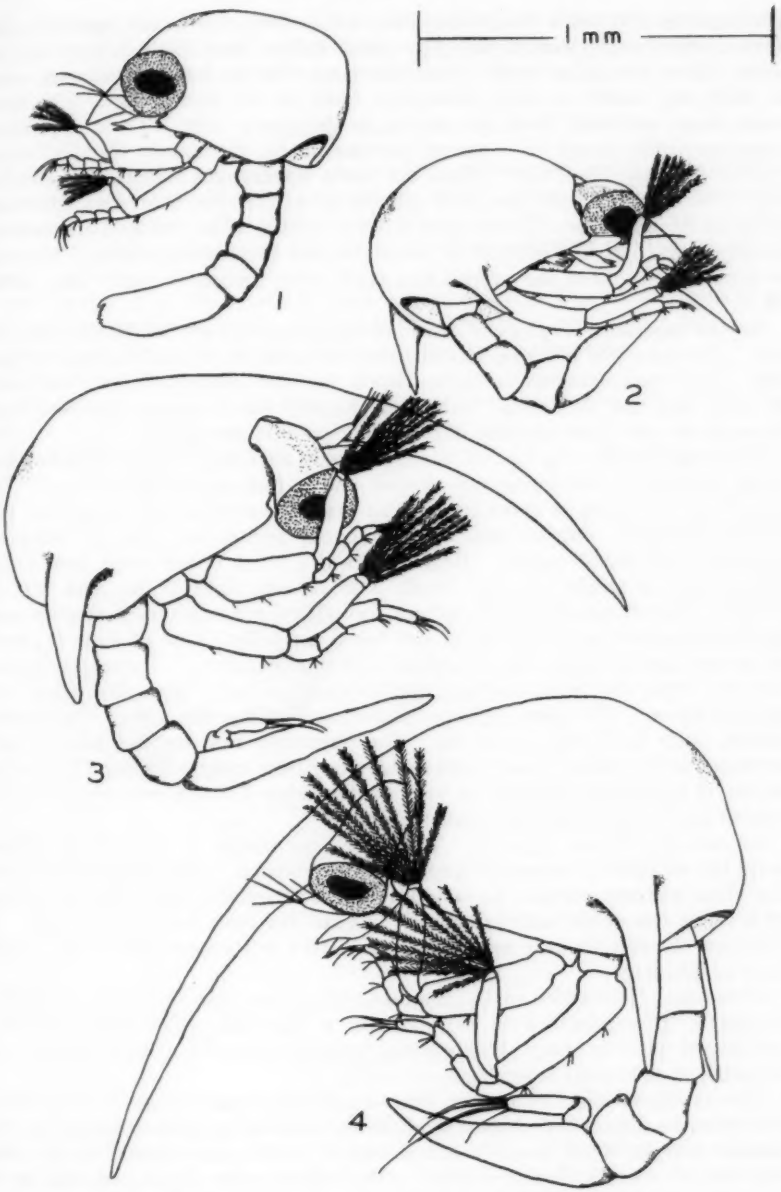


FIGURE 1. First zoea.
FIGURE 2. Second zoea.
FIGURE 3. Third zoea.
FIGURE 4. Fourth zoea.

right angle bend towards the median line so that their crowns are opposed. The crown is armed on its ventral edge by a stout, rather blunt tooth, followed by two shorter, sharp, triangular teeth. Next, there are three or four long slender, setae-like teeth and, finally, a sharp, triangular tooth on the dorsal edge. The entire crown slopes gradually from the ventral to the dorsal edge. These appendages change very little, except for a general increase in size, throughout the zoeal stages.

First maxillae (Fig. 22). These are fleshy appendages, adapted for handling food. The endopod bears three stiff setae at its tip and one short seta about halfway down its inner side. The exopod is twice as large as the endopod and flattened dorso-ventrally. It is divided at its distal end into two tapering horns. Part way down the outer side of the exopod is a small lobe, bearing a single long, curved seta.

Second maxillae (Fig. 24). Each of these appendages is divided into two parts. The protopod is a lobe, tapering anteriorly, where it bears a cluster of three setae. The scaphognathite is sickle-shaped, broader posteriorly than anteriorly, and very thin and foliaceous. Along its anterior-outer margin are nine setae. The posterior and inner margins of the scaphognathite are naked.

First maxillipeds (Fig. 1). These appendages are composed of a short coxopod, a long basipod, a two-segmented exopod and a four-segmented endopod. The basipod bears a group of three setae on its posterior margin just behind the joint with the endopod. One of these setae is shorter and stouter than the other two and armed with minute spines. Behind these is a group of two setae, then a short distance back, a single seta, and finally a single seta close to the joint with the coxopod. The endopod consists of four, cylindrical segments, each bearing setae. The first segment bears three setae just below the joint. One of these is shorter and stouter than the other two and armed with minute spines. The second segment bears two setae just below the joint, one of which is short, stout, and armed with spines as above. The third segment has two setae below the joint. The terminal segment bears four setae at its tip. The outermost two are the longest, curve downward at the ends and bear small spines along their inner margins. The exopod consists of a proximal segment as long as the endopod and a very short terminal segment which bears four long, plumose setae.

Second maxillipeds (Fig. 1). These are very similar to the first maxillipeds except the endopod is somewhat longer than the exopod. The basipod bears three setae along its inner margin; a group of two just behind the joint with the endopod and a single seta about halfway between this and the joint with the coxopod. No rudiments of other thoracic appendages are visible posterior to the second maxillipeds at this stage.

Abdomen. The abdomen is composed of five segments, the first of which is not clearly differentiated from the abdomen at this time. The sixth segment is consolidated with the telson; this becomes apparent when the uropods appear. No rudiments of abdominal appendages are visible.

The telson is slightly broader than long, and faintly concave. The lateral margins curve smoothly to a stout tooth at each side of the posterior margin. The posterior margin of the telson is armed with a complicated series of small spines, with minute denticles between them. The eighth spine from each side is the longest, and between these two longest spines are either nine or ten spines of intermediate length. Thus, in some cases there are twenty-five spines on the

posterior margin and in others there are twenty-six. This arrangement holds true for all the zoeal stages, there being sometimes twenty-five and sometimes twenty-six spines on the posterior margin of the telson.

Second Zoea (Fig. 2)

Two lateral spines are now present on the carapace, projecting posteriorly and downward. The rostrum has increased in length tremendously and is now as long as the carapace. The eyestalks are longer and the eyes are carried somewhat farther forward than in the first stage. The exopods of the maxillipeds bear six plumose setae.

Antennules (Fig. 9). Each of these appendages now bears a single stout seta instead of the three which were present in the first stage.

Antennae (Fig. 15). These are the same as in the first zoea.

Mandibles. As in the first zoea.

First maxillae. As in the first zoea except that the exopod bears three long teeth, the outer one showing no articulation at the base.

Second maxillae. As in the first zoea.

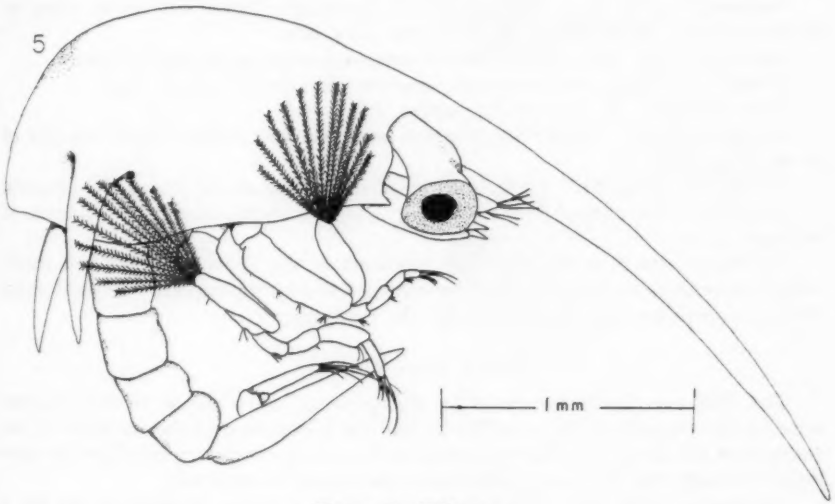


Figure 5. Fifth zoea.

Third Zoea (Fig. 3)

The shape of the carapace has changed somewhat. In the first zoea the carapace is practically hemispherical, in the second zoea it is less so, and now in the third zoea, its lateral outline is pear-shaped. The rostrum has continued to lengthen in comparison to the carapace and now exceeds the length of the carapace. Uropods appear on the telson. The exopods of the maxillipeds bear eight plumose setae.

Antennules (Fig. 10). Each of these bears three setae at its tip, much as in the first zoea stage.

Antennae (Fig. 16). As in the first and second zoea.

Mandibles. As in preceding stages.

First maxillae. As in the second zoea.

Second maxillae. As in the first and second zoea, the number of setae on the scaphognathite is nine.

Maxillipeds. The exopods bear eight plumose setae.

Uropods. These appear on the anterior ventral surface of the telson and consist of a short basal segment with a long, flattened lobe extending from it. This lobe is the exopod, as will be seen from later development, and bears two long setae at its tip.

The eyestalks have enlarged and project downward and forward. There is no evidence of any additional thoracic or abdominal appendages at this stage.

Fourth Zoea (Fig. 4)

This stage is characterized by the presence of ten plumose setae on the exopods of the maxillipeds and the fact that the uropod bears four setae on its exopod.

Antennules (Fig. 11). Each of these appendages bears four setae, three at the tip and one a short distance down on the inner side.

Antennae (Fig. 17). These are unchanged except for general growth.

Mandibles. These are somewhat slenderer than in preceding stages.

First maxillae. As in preceding stages.

Second maxillae. There are fourteen setae on the anterior-outer margin of the scaphognathite.

Maxillipeds (Fig. 4). These bear ten plumose setae at the tips of the exopods.

Uropods. The exopod bears two long and two short setae. No evidence of endopod as yet.

Abdomen. Each of the four free segments of the abdomen bears two small, round thickenings on its inner side, the evidence of future pleopods. No additional thoracic appendages are visible through the carapace.

Fifth Zoea (Fig. 5)

The fifth zoea is characterized by the presence of eleven or twelve plumose setae on the exopods of the maxillipeds, and the appearance of the rudiment of the endopod on the uropods. The rudiments of five future thoracic appendages are now visible through the carapace, posterior to the second maxillipeds.

Antennules (Fig. 12). Each bears six setae; a group of three at the tip, a group of two lower down on the inner margin and a single seta below these.

Antennae (Fig. 18). The rudiment of the flagellum is visible as a conspicuous knob, about half as long as the dentiform process, on the inner side of the antenna.

Mandibles. As in preceding stages.

First maxillae. As in preceding stages.

Second maxillae (Fig. 25). The number of setae along the anterior-outer margin of the scaphognathite has increased to nineteen.

Maxillipeds (Fig. 5). There are eleven or twelve plumose setae on the tips of the exopods. In the first four stages the number of setae on the exopods was

constant at four, six, eight, and ten, respectively; now there is some variation. Although twelve appears to be the more usual number, about one-third of the specimens examined had eleven setae on one or more of the maxillipeds. Individuals were found with twelve setae on the first maxilliped of the right side and eleven on the first maxilliped of the left side, and vice versa. This was also found to be true for the second maxillipeds. No individuals, in this stage of development, were found with less than eleven or more than twelve setae on the exopods of the maxillipeds.

Abdomen. As in the fourth zoea.

Uropods. The rudiment of the endopod now appears as a small bud below the exopod. The exopods have increased in length and each now bears five long setae at its tip.

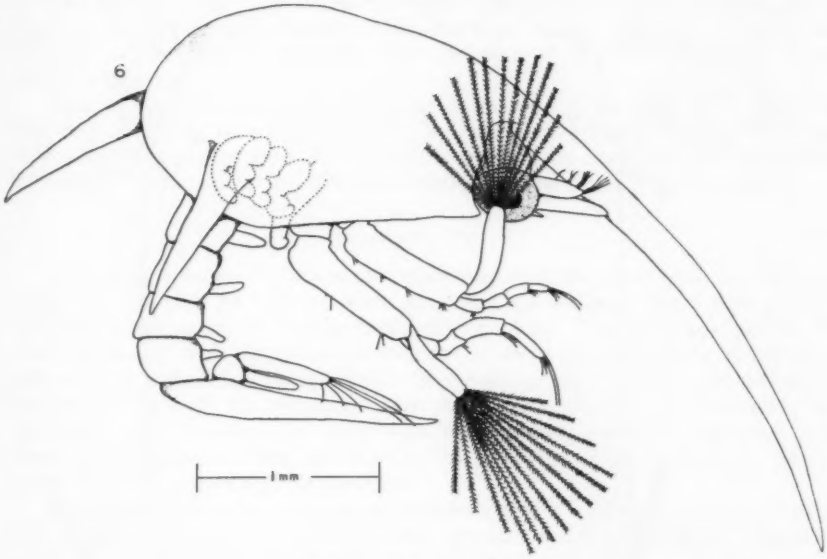


FIGURE 6. Sixth zoea.

SIXTH ZOEA (FIG. 6)

There are now uniramous pleopod buds on the four free segments of the abdomen. The exopods of the first and second maxillipeds bear thirteen or fourteen plumose seta. The rostrum continues to increase in length relative to the carapace and is one and one-half times the length of the carapace.

Antennules (Fig. 13). These appendages bear eleven setae in four groups: four at the tip, a group of four below this, a group of two below that, and finally a single seta below these.

Antennae. The flagellum has increased enormously, dwarfing the dentiform processes and extending well beyond the antennule.

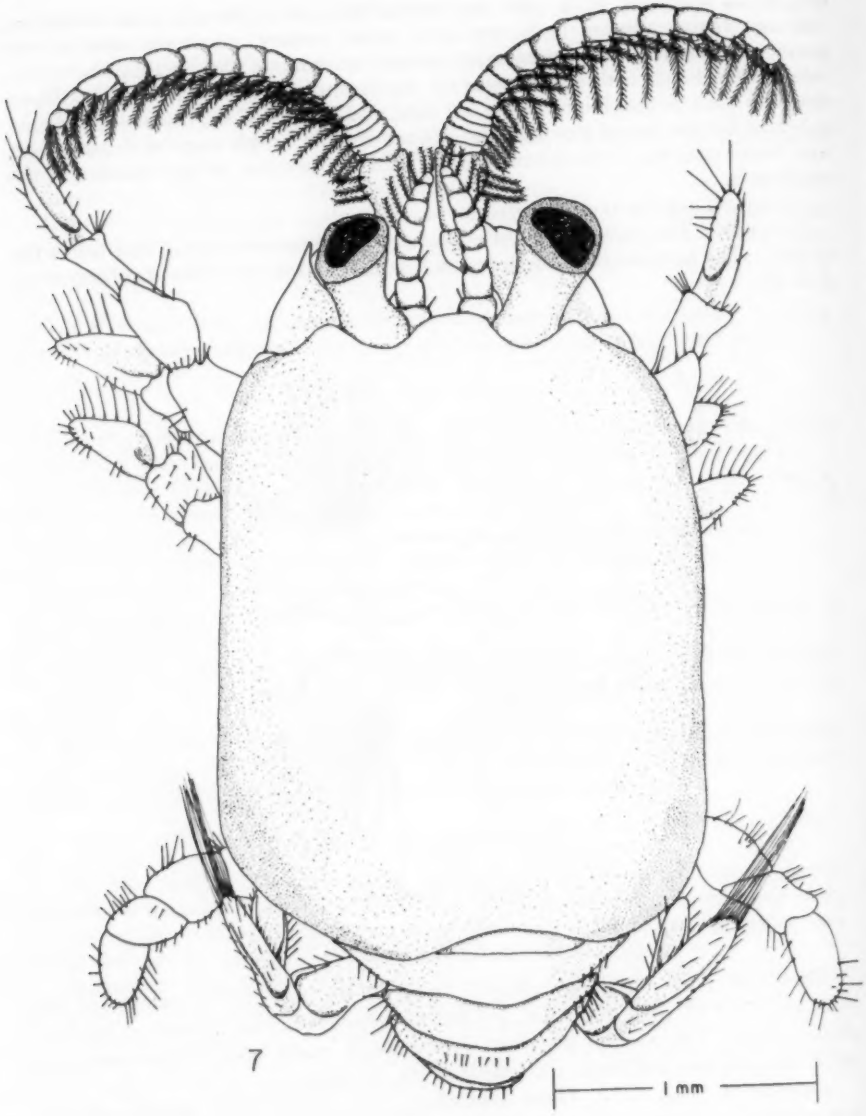


FIGURE 7. Megalops.

Mandibles (Fig. 21). The crown is armed much as in previous stages, but the shaft of the mandible is not so stout in proportion to its length.

First maxillae (Fig. 23). As in preceding stages.

Second maxillae. There are now 29 setae which extend all the way around the anterior-outer margin of the scaphognathite.

Abdomen. A pair of uniramous, unsegmented pleopods now appears on the second through the fifth segments of the abdomen.

Uropods. The endopod has increased considerably and is now two-thirds the length of the exopod. The exopod bears six setae of unequal length at its tip.

Maxillipeds (Fig. 6). The number of plumose setae on the exopods is now thirteen or fourteen occurring with about equal frequency. No individuals were found with less than thirteen or more than fourteen.

Five thoracic limb buds are plainly visible through the carapace. The first of these is the largest and extends below the edge of the carapace. This is the rudiment of the third maxilliped. The rudiment of the fifth pereiopod is not visible at this time.

Most of the individuals which became megalops did so at the molt following this stage. A few, however, went to a seventh zoea before becoming megalops. The only difference between this seventh zoea and the sixth was the appearance of additional setae on the maxillipeds, making the number fifteen or sixteen.

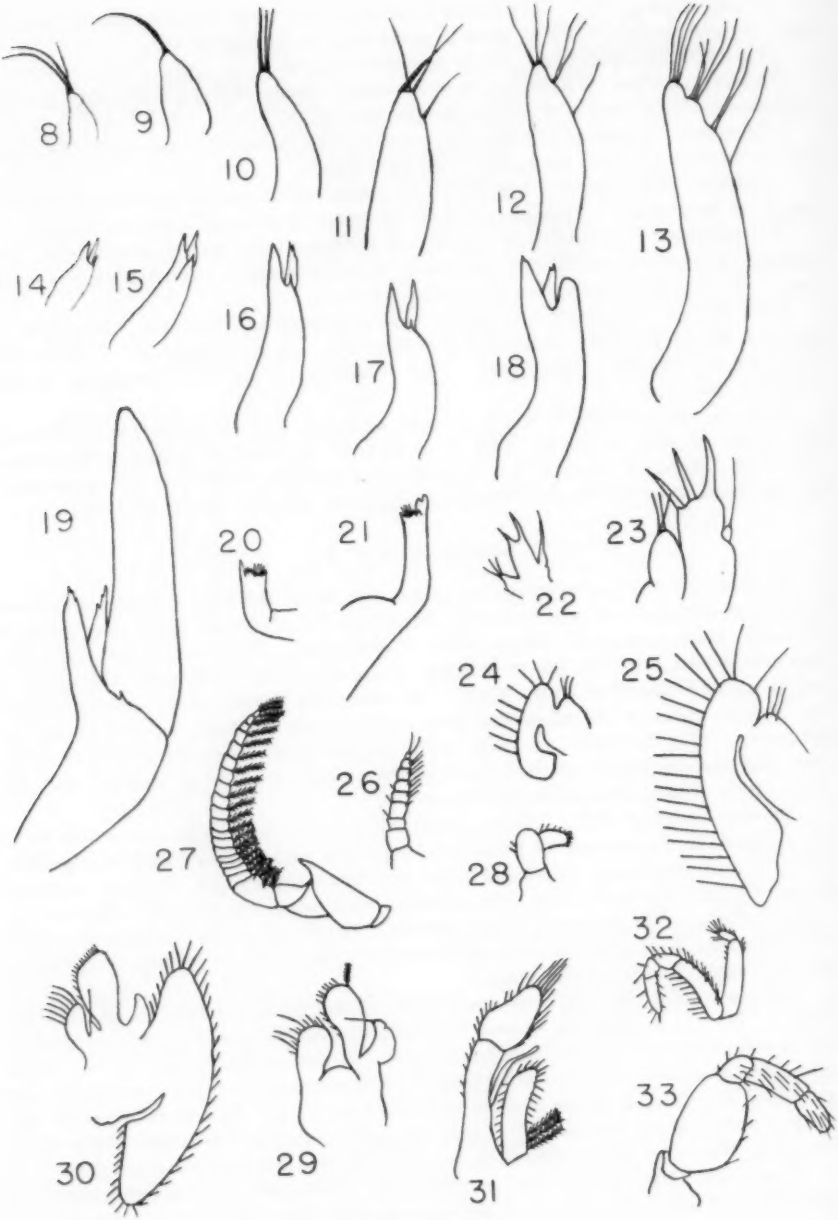
Megalops (Fig. 7)

In general form the megalops resembles the adult, the most obvious difference being that the eyes are still relatively large and the abdomen bears four pairs of pleopods which are quite unlike those of the adult. The megalops carries the abdomen flexed, with the telson between the bases of the pereiopods, though it is not as strongly flexed as in the adult. In swimming the megalops sometimes, but not always, extends the abdomen, thus utilizing the pleopods as swimming appendages. This tendency to swim with the abdomen extended decreased with time and individuals which had been in the megalops stage as long as one day were rarely seen to extend the abdomen.

Antennules (Fig. 26). These appendages are now as long as the peduncles of the antennae and composed of three basal segments and a six-segmented flagellum, which is not noticeably delineated from the basal segments. The first segment bears one, and the succeeding five segments each bear two plumose setae on the outer margin. The segments of the peduncle and the two lower segments of the flagellum may bear a single seta on the inner side. The secondary flagellum of the adult is not present at this stage.

Antennae (Fig. 27). These now possess all the important features of the adult form. There is a scale-like exopod and an endopod composed of three segments and a long flagellum. The flagellum is stout, tapers gradually to a rather blunt tip, and is composed of eighteen segments. The segments are short proximally but gradually increased in length distally until they are longer than broad near the tip. Each segment bears four setae. There are two long, plumose setae which curve inward at their tips, and within these are two shorter, straight, unarmed setae.

Mandibles (Fig. 28). The mandible has undergone a complete change in structure and function. It is no longer an organ of mastication but is adapted, as in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FIGURES 8–33.

the adult, for the purpose of scraping the antennae and passing food to the mouth. The mandible is now composed of two parts, a broad, foliaceous outer lobe bearing two short, stout setae on its lateral margin, and an inner palp fringed with setae along its anterior and median margins. A very noticeable difference between the appendages of the megalops and those of the adult is the relatively sparse setation of the megalops appendages. In the adult all the appendages are densely fringed with setae.

First maxillae (Fig. 29). The first maxillae now possess all the parts of the adult appendage. The inner lobe (endopod) is broad, bluntly rounded at the tip and armed with stout setae of varying length around the tip and part way down the inner margin. The outer lobe (exopod) is longer and much narrower at the base but broad and round at the tip. It is armed with short, stout setae at the tip and along the inner margin. There is one long plumose seta at the beginning of the outer margin. The palpus is a sac-like lateral projection near the base of the inner lobe. It bears a single long seta at its tip.

Second maxillae (Fig. 30). These appendages are like the adult form except for the relative proportions of the endites and the sparsity of setae as compared to the adult. In the megalops the endites compose three lobes, a small inner lobe bearing long curved setae down its inner margin, a much larger outer lobe fringed with short setae along its anterior-inner margin, and a small papiliform lobe between these two, bearing a single long seta. Between the outer endite and the scaphognathite is a small, triangular lobe representing the endopod. The scaphognathite is broad, thin and has much the same form as in the zoeal stages. It now tapers more acutely anteriorly and the fringe of setae has extended around the broad posterior margin.

First maxilliped (Fig. 31). The anterior segment of the protopod is elongated into a flat, blade-like process bearing setae around its margins, with a series of much longer, plumose setae on the posterior portion of the inner margin. The endopod is represented by a soft, slender lobe arising from near the base of the inner side of the two-segmented exopod. The exopod consists of a long basal segment bearing short setae along its outer margin, and a shorter, broader, paddle-shaped terminal segment bearing very long setae at the tip and shorter setae along its other margins.

Second maxillipeds (Fig. 32). The four-segmented endopod of the second maxilliped differs slightly from the adult form. The third segment makes a right angle bend in the adult, while it is practically straight in the megalops, and the terminal segment is proportionally much shorter and stouter in the megalops than in the adult. The exopod is two-segmented; the basal segment does not taper anteriorly as acutely as in the adult and the oval terminal segment is proportionally smaller in the megalops than in the adult. As in previous cases, the adult appendage is very heavily fringed with setae, while in the megalops the setae are shorter and much sparser.

FIGURES 8–13. Antennule, first to sixth zoea.
FIGURES 14–19. Antenna, first to sixth zoea.
FIGURE 20. Mandible, first zoea.
FIGURE 21. Mandible, sixth zoea.
FIGURE 22. First maxilla, first zoea.
FIGURE 23. First maxilla, sixth zoea.
FIGURE 24. Second maxilla, first zoea.
FIGURE 25. Second maxilla, fifth zoea.
FIGURE 26. Antennule, megalops.
FIGURE 27. Antenna, megalops.
FIGURE 28. Mandible, megalops.
FIGURE 29. First maxilla, megalops.
FIGURE 30. Second maxilla, megalops.
FIGURE 31. First maxilliped, megalops.
FIGURE 32. Second maxilliped, megalops.
FIGURE 33. Third maxilliped, megalops.

Third maxillipeds (Fig. 33). These broad, opercular appendages bear a three-segmented palp at their distal end. The rounded prominence at the articulation of the palpus is lacking in the megalops. The palpus is stouter and the terminal segment much shorter than in the adult.

Pereiopods. The pereiopods are so much like those of the adult that a detailed description of them is unnecessary. The first, second, and third pairs project anteriorly; the fourth pair projects posteriorly and, like the first three pairs, is especially adapted for burrowing in the sand. The fifth pair of pereiopods are very slender, chelate appendages, which are held concealed within the branchial cavity.

Abdomen. The abdomen is, for the first time, composed of six segments, similar in form and proportion to those of the adult, and a triangular telson. The first segment, as seen dorsally, is a small plate, filling a curved sinus in the posterior margin of the carapace. The second segment is the largest of the abdominal segments and is about five times as wide as it is long. Its width is due to a broad lamellar expansion on each side. The third, fourth, and fifth segments are rounded at the outer margins and each is slightly shorter and narrower than the one before. The sixth segment is nearly as wide as the fifth and is as long as it is wide, being the longest of the abdominal segments. The second, third, fourth, and fifth segments of the abdomen each bear a pair of biramous pleopods. The pleopods are made up of three portions; a long basal segment, a paddle-shaped exopod and a knob-like endopod. The pleopods of the second and third segments bear ten plumose setae each on the exopods and those of the fourth and fifth segments bear eleven plumose setae on the exopods. The small endopods increase in length successively from the first to the fourth pleopod, and bear at their tips a series of small hooks. These hooks can engage those of the endopod opposite it, thus joining the pair of pleopods so that they move as one.

Uropods. The appendages of the sixth abdominal segment are essentially the same as those of the adult. There is a two-segmented protopod, the proximal segment of which is short and round and the distal segment, much longer, stouter and flattened. The exopod and endopod are nearly alike: oval, broadly rounded at the tip and fringed with setae which are very long at the tips but shorter along the sides.

Discussion

The first zoea of *Emerita talpoida*, obtained from the egg, corresponds with the first zoea described by Faxon (1879a). The larvae described by Smith (1877) from the plankton as the second, third, and last zoea do not correspond exactly with any of the zoeal stages reared in the laboratory. The results of the present experiments indicate that each zoeal molt results in an increase in the number of setae borne on the exopods of the first and second maxillipeds. In the laboratory their number never increased by more than two setae at any one molt. Using the number of setae on the maxillipeds as an indication of the number of times that an individual has molted, Smith's three zoeae correspond to the third, fourth, and fifth zoeal stages reared in the laboratory. In each case, however, the zoeae from nature possess features (appearance of thoracic limb buds, pleopods, etc.) which show them to be farther advanced in development than the corresponding laboratory stages.

Smith's last zoeal stage bears twelve setae on the exopods of the first and second maxillipeds. A number of individuals in this stage were observed by Smith to change to megalops at a single molt. If setation of the maxillipeds is an accurate index to the number of zoeal molts, then these individuals from nature completed their larval development in five molts, whereas those in the laboratory went through six or seven molts before becoming post-larvae.

Gurney (1942) has questioned the normality of larvae reared in the laboratory and believes that abnormal stages may be reared under artificial conditions. However, Gurney also states that extra stages, through which each individual need not pass in development, occur in nature. Other references to these extra stages have been made by Faxon (1879b), Lebour (1940), Gurney and Lebour (1941), and Broad (1957a). Johnson and Lewis refer to a "Lower Stage IV" in the larvae of *E. analoga*, which was intermediate between Stage III and Stage IV. They could not say whether this was a distinct instar or simply a variable in Stage IV. This is apparently another case of a stage in larval development through which not all individuals pass. If each larval form found in the development of a decapod is described as a stage, then we may expect to find individuals who skip stages or pass through extra stages in the course of normal development, depending upon the number of stages previously defined.

Broad (1957b) working with *Palaemonetes* larvae, has shown that a direct relationship exists between the diet of the larvae and the rate of larval development and frequency of molting. His results show that larvae may respond to suboptimal conditions of diet by a prolonged larval life and a greater number of larval intermolts. It is probable that a number of other environmental factors also affect the tempo of larval development, and that normal development of *Emerita* and other decapods varies according to the variations in trophic conditions during the breeding season.

Summary

1. *Emerita talpoida* were reared in the laboratory from eggs to the megalops. Of individuals hatched in the laboratory 15 per cent survived to the megalops.

2. The average length of time required to pass through the pelagic larval stages in the laboratory was 28 days. A table is given which shows the average duration of each of the zoeal stages.

3. Six zoeal stages and a megalops are figured and described. A seventh zoeal stage, which was skipped by most of the larvae, is described.

4. Larvae reared in the laboratory are compared with three zoea stages described from the plankton by Smith.

5. The larval development of *Emerita talpoida* should not be regarded as consisting of a fixed number of stages determined by a fixed number of larval intermolts.

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FREE AMINO ACIDS IN SOME AQUATIC INVERTEBRATES[1]

JOHN W. SIMPSON,[2] KENNETH ALLEN AND JORGE AWAPARA

Department of Biology, The Rice Institute, Houston, Texas

Free amino acids and many of their derivatives are found present in tissues of all invertebrates so far studied. The distribution of amino acids seems to follow a pattern characteristic for the species. Compounds such as taurine were found in very high concentration in a number of invertebrates. As early as 1904, Kelly reported that *Mytilus edulis* has as much as 1.6 per cent taurine. Other invertebrates contain also large amounts of taurine (Henze, 1905; Mendel, 1904; Kossel and Edlbacher, 1915; Okuda, 1920; Ackermann *et al.*, 1924; Ackermann, 1935; Lewis, 1952; and Kermack *et al.*, 1955). The function of taurine in invertebrates is not known at all, and its mode of formation remains to be elucidated.

The distribution of free amino acids in a number of invertebrates has been studied by Camien *et al.* (1951), Duchâteau and Florkin (1954), Duchâteau *et al.* (1952), and Giordano *et al.* (1950). The amino acids were determined microbiologically. Camien *et al.* found very high concentrations of glycine in muscles of *Homarus vulgaris* and *Maia squinado* and suggested that the role of glycine along with other amino acids was to regulate osmotic pressure. Kermack, Lees and Wood (1955) made an extensive study of the non-protein constituents of the lobster. They found that a large portion of the non-protein nitrogen was accounted for as free alpha-amino nitrogen. The remainder was distributed between trimethylamine oxide, glycine betaine, taurine and volatile bases.

The object of the present study was to determine the pattern of distribution of free amino acids and related substances and to establish a correlation between the pattern of distribution and species and/or environment. The results reported here indicate that such differences do exist in relation to environment and species.

Materials and Methods

Animals investigated

The invertebrates investigated include representatives of the following phyla: Coelenterata, Arthropoda, Mollusca, and Echinodermata. The specimens were taken from their environment, rapidly frozen and maintained in this state just prior to analytic procedures (not more than a one-month period). Table I lists these organisms according to phylum and class and shows the location and habitat from which the specimens were taken.

Extraction

Immediately after thawing, the whole organism was quickly weighed and the nitrogenous substances extracted with 80 per cent ethanol according to the pro-

[1] This work was supported by grants from the Robert A. Welch Foundation, Houston, Texas, and the National Institutes of Health, U. S. Public Health Service.

[2] Part of these data was taken from the M.A. Thesis of John W. Simpson.

TABLE I

Organisms studied and their habitat

Organism*	Location of collection	Habitat
Coelenterata		
Anthozoa		
Bunodosoma cavernata	PA	W
Arthropoda		
Crustacea		
Penaeus aztecus	AB	W
Clibinarius vittatus	SLP	E
Pagurus pollicaris	AB	W
Mollusca		
Gastropoda		
Oliva sayana	SLP	E
Polinices duplicata	AB	W
Thais haemastoma haysae	AB	W
Busycon perversum	AB	W
Fasciolaria distans	PA	W
Siphonaria lineolata	PA	E
Pelecypoda		
Lithophaga bisulcata	PA	W
Crassostrea virginica	SLP	W
Arca umbonata	PA	W
Volsella demissus granosissimus	EBL	E
Cephalopoda		
Loliguncula brevis	AB	W
Echinodermata		
Holothuroidea		
Thyone sp.	SLP	W
Asteroidea		
Luidia clathrata	SLP	W

PA—Port Aransas, Texas
AB—Aransas Bay, near Rockport, Texas
SLP—San Luis Pass, Galveston Island, Texas
EBL—East Beach Lagoon, Galveston Island, Texas
W—Collected directly from marine waters
E—Collected during a period of exposure

* We are indebted to Mr. Howard Lee from the Texas Game and Fish Commission, Rockport, Texas, for permitting the use of equipment necessary for acquisition of organisms.

cedure of Awapara (1948). The amino acids extracted are not produced during the extraction procedure by proteolytic cleavage. Extractions were carried out using live organisms under conditions such as to prevent any enzymatic activity. The live organisms were ground with 80 per cent ethanol as indicated above. Others were frozen and then thawed. Extracts were also prepared as described. Analysis of the extracts revealed that no change had occurred as a result of freezing and thawing.

Fractionation of extractives

Using ion-exchange chromatography the nitrogenous extractives were separated into basic, acidic and neutral substances. Basic substances were separated from neutral and acidic substances on Amberlite CG-50, type 2 H^+, with a screen grading of approximately 200 (passing 200 mesh). The tissue extracts were placed in a beaker containing one gram of the resin and shaken for thirty minutes to allow equilibration with the resin. This suspension was placed in a small column

TABLE II

Amino acids identified

Organism	Al	B-Al	Gly	Ar	As	Glu	Tau	Gla	Pr	OH-Pr	Thr	Tyr	Aspr	His
Coelenterata														
Anthozoa														
Bunodosoma cavernata	+	−	+	+	+	+	+	+	−	−	−	−	−	−
Arthropoda														
Crustacea														
Penaeus aztecus	+	+	+	+	+	+	+	+	+	−	−	+	+	+
Clibinarius vittatus	+	−	+	+	+	+	+	+	+	−	+	+	+	+
Pagurus pollicaris	+	−	+	+	+	+	+	+	+	−	+	+	+	+
Mollusca														
Gastropoda														
Oliva sayana	+	−	+	+	+	+	+	+	−	−	−	−	−	−
Polinices duplicata	+	−	+	+	+	+	+	+	+	+	−	−	−	−
Thais haemastoma haysae	+	−	+	+	+	+	+	+	+	+	−	+	+	+
Busycon perversum	+	−	+	+	+	+	+	+	+	+	−	−	+	+
Fasciolaria distans	+	−	+	+	+	+	+	+	−	−	+	+	+	+
Siphonaria lineolata	+	−	+	+	+	+	+	+	+	+	−	+	+	−
Pelecypoda														
Lithophaga bisulcata	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Crassostrea virginica	+	+	+	+	+	+	+	+	+	−	+	−	−	−
Arca umbonata	+	+	+	+	+	+	+	+	+	−	+	−	−	+
Volsella demissus granosissimus	+	+	+	+	+	+	+	+	+	−	+	+	−	+
Cephalopoda														
Loliguncula brevis	+	+	+	+	+	+	+	+	+	+	+	−	−	−
Echinodermata														
Holothuroidea														
Thyone sp.	+	−	+	+	+	+	+	+	−	−	−	−	−	−
Asteroidea														
Luidia clathrata	+	−	+	+	+	+	+	+	−	−	−	−	−	−

Legend:

\+ Present in readily detectable amounts
− Not present in readily detectable amounts

Al—alanine
B-Al—beta alanine
Gly—glycine
Ar—arginine
As—aspartic acid
Glu—glutamic acid
Aspr—asparagine
Tau—taurine
Gla—glutamine
Pr—proline
OH-Pr—hydroxyproline
Thr—threonine
Tyr—tyrosine
His—histidine

(20 × 1 cm.) containing an additional gram of the resin. The neutral and acidic compounds were washed from the resin with 25 cc. water and the basic substances were eluted from the column with 25 cc. 4 N HAc. (Awapara, Davis and Graham, 1959). After removal of the basic substances, taurine and other sulfonic acids were separated from the neutral and acidic amino acids on a column of Dowex-50 H^+. The water wash from the Amberlite was passed slowly through a column containing one gram of Dowex-50 H^+. Taurine was obtained by washing with 25 cc. water and the neutral and acidic amino acids eluted from the resin with 25 cc. 4 N NH_4OH. Each fraction was evaporated to dryness on a steam bath and brought to a 1-cc. volume with water.

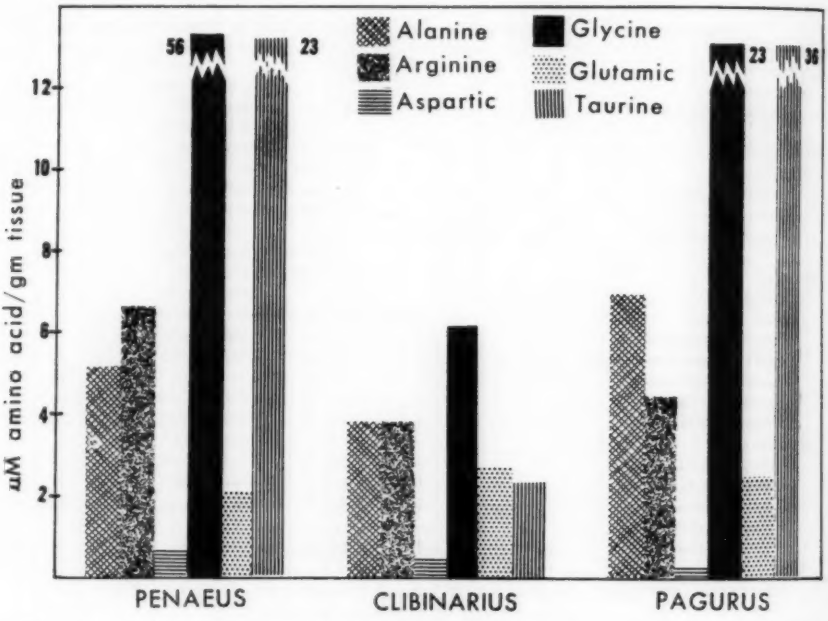

FIGURE 1. Estimated concentration of six amino acids in the tissues of three crustaceans: *Penaeus aztecus, Clibinarius vittatus* and *Pagurus pollicaris.*

Paper chromatography

Amino acids of the acidic and neutral fractions were separated by two-dimensional ascending paper partition chromatography. Whatman number 3 MM. filter paper was used and the solvent system employed was phenol-water (72.5 per cent phenol) and 2,4-lutidine-water (62 per cent) in the second direction. The basic amino acids were separated by one-dimensional ascending paper partition chromatography using butanol, acetic acid and water (4 : 1 : 1, by volume) as the solvent.

The amino acid spots were revealed by dipping the paper in a solution of 0.5 per cent ninhydrin in absolute ethanol (w/v).

Identification of amino acids

Amino acids were identified by: 1) their ninhydrin color and position on chromatograms according to previously prepared maps of known substances, and 2) their presence in a particular fraction.

Arginine was further identified by the Sakaguchi reaction, using the alpha napthol reagent described by Acher and Crocker (1952), and histidine by the Pauly's reaction using the sulfanilic acid reagent described by Smith (1958).

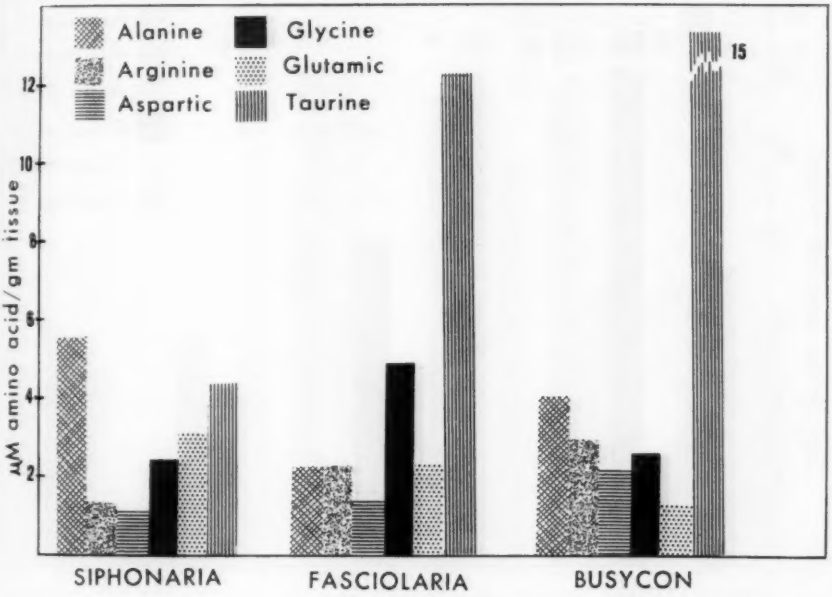

FIGURE 2. Estimated concentration of six amino acids in the tissues of three gastropods: *Siphonaria lineolata*, *Fasciolaria distans* and *Busycon perversum*.

The identification of β-alanine and taurine was supported by preparing chromatograms on paper treated with basic cupric carbonate (Block *et al.*, 1958). These two substances moved to their respective positions on both treated and untreated papers.

Glutamine was further identified by subjecting an aliquot of the extract to hydrolysis with 6 N HCl. Chromatograms of the hydrolysed extracts showed the disappearance of this spot; further, these same chromatograms showed an increase in the glutamic acid spot.

The identification of the asparagine spot was substantiated by elution from undeveloped chromatograms with subsequent acid hydrolysis and re-chromatography of the eluate. Chromatograms of the hydrolysate showed the absence of the spot and the appearance of a spot which corresponded in position and color with the aspartic acid standard.

Estimation of amino acids

Six amino acids which were consistently present were estimated quantitatively. Alanine, aspartic acid, glycine and glutamic acid were measured by the method of Awapara, Landua and Fuerst (1950) with the following modifications suggested

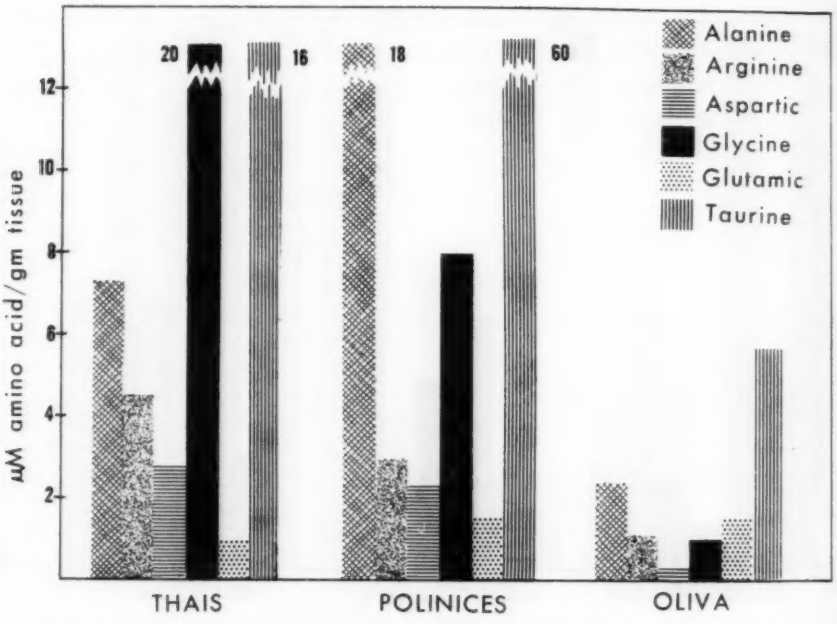

FIGURE 3. Estimated concentration of six amino acids in the tissues of three gastropods: *Thais haemastoma, Polinices duplicata* and *Oliva sayana.*

by Fowden (1951): 1) the areas of paper containing the spots were cut, placed in test tubes, and 0.2 cc. N NaOH added. The tubes were evacuated for three hours in a desiccator containing concentrated H_2SO_4; 2) additional citrate was included in the ninhydrin reagent of Moore and Stein (1948) equivalent to the amount of NaOH added.

Taurine was found to be the only ninhydrin-positive material present in the acidic fraction; therefore, this substance was measured directly using the color reagent of Moore and Stein (1948).

Arginine was measured in the extracts by the method of Rosenberg *et al.* (1956). We found no other Sakaguchi-positive material in the extracts as determined by paper chromatography. If other guanidine derivatives were present, their concentration was too low to measure. We can safely assume that nearly all the color in the Rosenberg reaction was due to arginine .

Results and Discussion

In Table II are shown all the amino acids and related substances detected chromatographically. Other components were present but their identity was not established. In Figures 1 to 6 are shown the concentrations of six amino acids

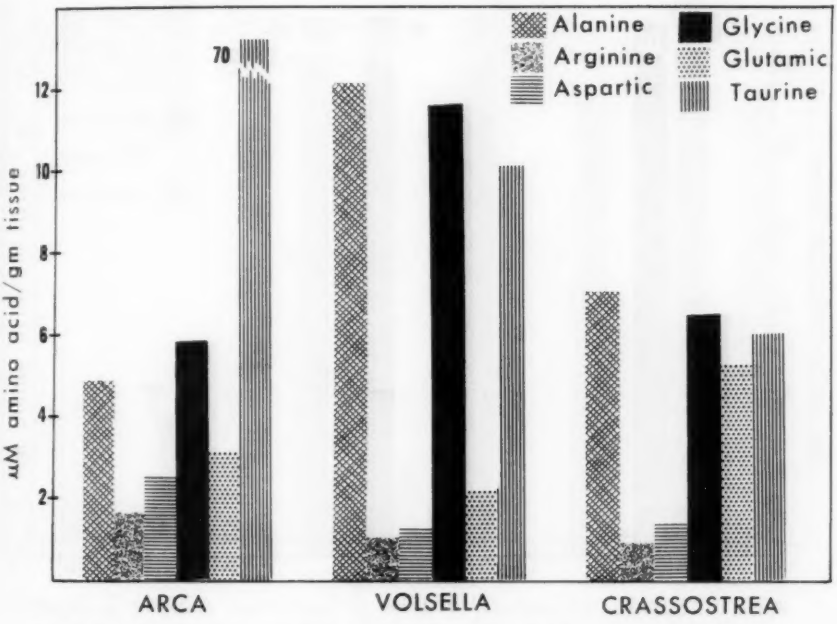

Figure 4. Estimated concentration of six amino acids in the tissues of three pelecypods: *Arca umbonata*, *Volsella demissus granosissimus* and *Crassostrea virginica*.

which were found invariably in the species studied. The most striking feature is the high concentration of taurine and glycine in nearly all species studied. The glycine concentration is in many cases equal to that of taurine. The highest concentration of taurine was found in *Penaeus aztecus*, but our value is still considerably lower than the value reported for *Mytilus edulis*. The mode of formation of taurine in invertebrates is not known at all. In mammals it is formed from cysteine after oxidation to cysteine sulfinic acid and decarboxylation of the latter

to hypotaurine (Awapara and Wingo, 1953). Hypotaurine is then oxidized to taurine. Hypotaurine has been reported only once in invertebrates (Shibuya and Ouchi, 1957). The cysteine sulfinic decarboxylase has not been reported in invertebrates. There is the possibility that taurine is not produced by invertebrates but acquired from their diet. Glycine, which is also present in very high amounts, could also be obtained from their diet. One objection to this possibility is the wide range of variation in the taurine concentration of species from the same environment.

Aspartic acid, glutamic acid, and alanine vary much less in concentration than taurine and glycine.These three amino acids are closely linked to the citric acid

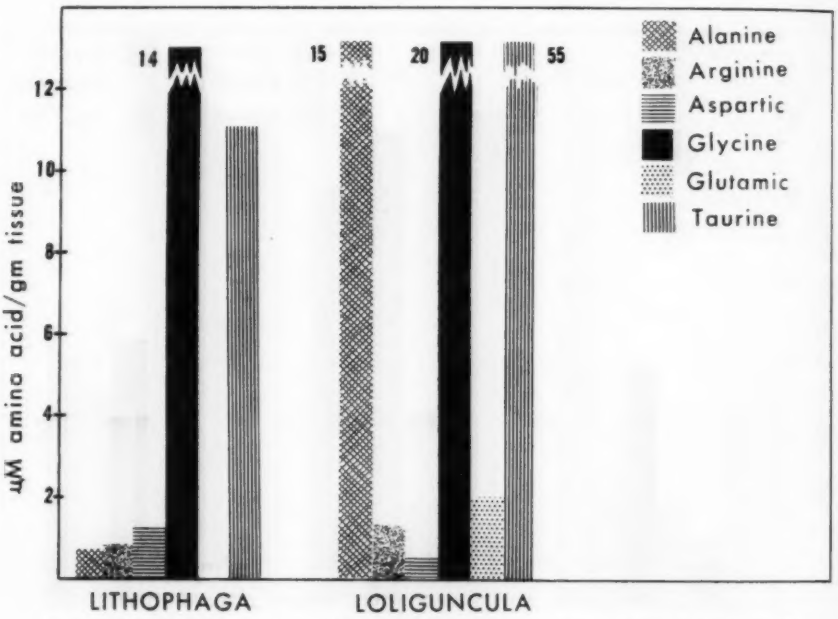

FIGURE 5. Estimated concentration of six amino acids in the tissues of pelecypods *Lithophage bisulcata* and the cephalopod *Loliguncula brevis*.

cycle and their concentration could be easily regulated by the reactions of the cycle. β-alanine was found in some forms but not in others. The only coelenterate which we studied had none. It was found in some Mollusca but not in others. The same was true in the arthropods studied. The function of β-alanine is again unknown. It exists usually as a moiety of carnosine which is known to exist in the muscle of vertebrates and many invertebrates. The problem of formation arises again. β-alanine can be produced by the decarboxylation of aspartic acid, or by the hydrolytic breakdown of dihydrouracil. Similarly, β-aminoisobutyric acid is formed by the hydrolytic breakdown of dihydrothymine. We found β-aminoisobutyric acid in

small amounts in *Volcella* and in larger amounts in *Mytilus*. A somewhat curious finding is that of asparagine in certain representatives of the Mollusca and Arthropoda, as shown in Table II. Asparagine is not often found in detectable amounts in mammals.

Arginine was present in all the species studied. It probably resulted from the hydrolysis of arginine phosphate, a well-known phosphagen in invertebrates.

Inasmuch as the taurine concentration appeared to vary with the environment we proceeded to study a number of fresh-water forms and also other marine forms for their taurine content. The results are shown in Table III. The presence of

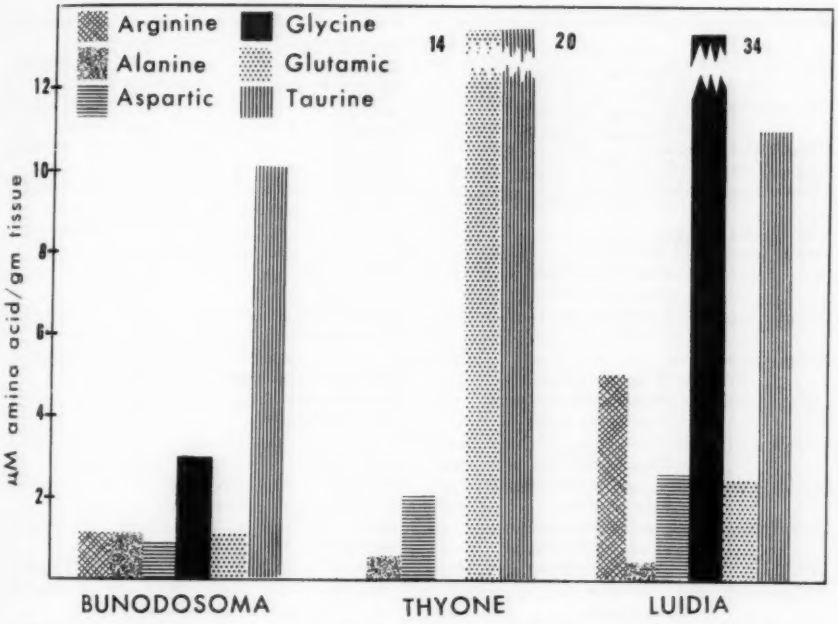

FIGURE 6. Estimated concentration of six amino acids in the tissues of the coelenterate *Bunodosoma cavernata* and the echinoderms *Thyone* sp. and *Luidia clathrata*.

taurine by chromatography is detectable in concentrations as low as 0.1 micromole per gram. Our results indicate that taurine is either absent in the fresh-water forms of molluscs or it is present in concentrations lower than 0.1 micromole per gram. Every fresh-water and terrestrial mollusc studied showed no taurine whereas every form of invertebrate living in salt water or brackish water contained taurine in detectable and measurable amounts.

At this stage of this study, it is not possible to attribute to taurine any definite role. But the evidence is suggestive that it plays a very important role as an osmoregulator.

TABLE III

Taurine content in various molluscs

Mollusca	Environment	Taurine
Gastropoda		
Lymnaea palustris	Fresh water	–
Marisa cornuarietis	Fresh water	–
Pomacea bridgesi	Fresh water	–
Rumina decollata	Terrestrial	–
Otala lactea	Terrestrial	–
Mesodon thyroidus	Terrestrial	–
Bulimulus alternatus	Terrestrial	–
Murex fulvescens	Marine	+
Littorina irrorata	Marine	+
Oliva sayana	Marine	+
Polinices duplicata	Marine	+
Busycon perversum	Marine	+
Siphonaria lineolata	Marine	+
Fasciolaria distans	Marine	+
Thais haemastoma haysae	Marine	+
Pelecypoda		
Anadonta grandis	Fresh water	–
Quadrula quadrula	Fresh water	–
Lampsilis sp.	Fresh water	–
Elliptio sp.	Fresh water	–
Rangia cuneata	Brackish-Fresh water	+
Brachiodontes recurvus	Brackish-Marine	+
Crassostrea virginica	Brackish-Marine	+
Donax variabilis	Marine	+
Venus mercenaria	Marine	+
Dosinia discus	Marine	+
Arca incongrua	Marine	+
Arca campechiensis	Marine	+
Noetia ponderosa	Marine	+
Cephalopoda		
Loliguncula brevis	Marine	+

SUMMARY

1. Free amino acids of 17 species of aquatic invertebrates were determined by chromatographic methods.

2. Qualitative differences in some amino acids were detected in organisms of different species. The concentration of alanine, arginine, aspartic acid, glutamic acid, glycine and taurine was measured and significant differences recorded.

3. Taurine was found in high concentration in all the marine organisms studied but was not found in several fresh-water and terrestrial organisms.

4. The possible role of taurine and other free amino acids in aquatic organisms is discussed.

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ABSTRACTS OF PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY 1959

ABSTRACTS OF SEMINAR PAPERS

JULY 7, 1959

Isolation and chemical identification of the crystalline cytoplasmic inclusions in the large, free-living amebae. JOE L. GRIFFIN.

The crystalline inclusions of *Amoeba proteus*, *Amoeba dubia*, and *Chaos chaos* have been shown to be composed of the same substance. This crystalline material has been isolated, purified, and shown to be identical with synthesized carbonyl diurea, with regard to physico-chemical properties, elemental analysis, x-ray diffraction patterns, infra-red spectra and optical properties. The two types of crystals found in the above species, plates and bipyramids, are apparently alternative crystalline arrangements of carbonyl diurea. It is suggested that carbonyl diurea represents an end product of nitrogen metabolism in amebae.

Physico-chemical characterization of chromatophorotropins in the crayfish Cambarellus shufeldti. MILTON FINGERMAN.

Sinus glands and central nervous organs were observed with an electron microscope. These organs contained neurosecretory granules that were not bounded by a membrane. Organs containing these granules were extracted in hypotonic and hypertonic media. The hypertonic extract was more potent in concentrating red pigment. This result would not have occurred if the granules were bounded by a semipermeable membrane. Extracts of eyestalks and supraesophageal ganglia plus the circumesophageal connectives were incubated with trypsin for one hour at 33° C. The chromatophorotropins were almost completely inactivated. Presumably the chromatophorotropins contain peptide bonds. These hormones are not inactivated by boiling. Extracts of the organs were then subjected to filter paper electrophoresis for two hours at pH 7.6–7.7. The dark red pigment dispersing hormone in the eyestalks and the concentrating hormone in the supraesophageal ganglia and circumesophageal connectives were electropositive. The dispersing substance in the latter two organs was electronegative. At pH 2.3 the dispersing hormone in the supraesophageal ganglia and circumesophageal connectives was electropositive; its charge had been reversed. The isoelectric point is probably about pH 4.0. When electrophoresis was performed at pH 8.9 some of the molecules of dispersing hormone in the eyestalk and of the concentrating hormone in the supraesophageal ganglia and circumesophageal connectives remained at the origin and some were electronegative. The isoelectric point of both substances is probably near pH 8.5. These chromatophorotropins are presumably polypeptides since their charge can be reversed, they are heat-stable, and they can be inactivated by trypsin.

This investigation was supported by Grant No. B-838 from the National Institutes of Health.

The molecular basis for the "catch" mechanism in molluscan muscles. WILLIAM H. JOHNSON AND ANDREW G. SZENT GYÖRGYI.

It has been known for some time that certain muscles, such as the adductor muscles of bivalves, are capable of remaining contracted for considerable periods of time without much apparent utilization of energy. This paradox has been attributed by some to tetanic contrac-

tions of high efficiency; others have suggested that a change in the visco-elastic properties of the muscle occurs along with contraction which is capable of maintaining tension or shortening without requiring much energy. Evidence favoring the second type of mechanism has been obtained by one of us. Investigation of the properties of paramyosin, a protein found in large amounts in these muscles, has revealed a critical dependence of solubility upon the pH and ionic strength of the medium. Similar dependence on these parameters is seen when glycerinated fibers prepared from these muscles contract without load in ATP. These results suggested that paramyosin might be a key part of a system which determines the mechanical properties of the "catch muscles." To test this possibility, glycerinated fibers prepared from the anterior byssus retractor muscle of *Mytilus edulis*, a muscle which shows the above described behavior, were stretched and the tension thus developed measured under conditions where the actomyosin system should contribute little to the stiffness of the fiber. Salyrgan (10^{-4} *M*) and 10^{-2} *M* pyrophosphate were added to the medium. Measurements of fiber stiffness were made at various values of pH and ionic strength. At low ionic strength (0.07), fibers were relatively stiff at pH values below 6.5, a range in which paramyosin crystallizes out of solution, while at pH values above this, the fibers were relatively plastic. Thus the extensibility of the fibers parallels the solubility of paramyosin, and, since paramyosin is present in rodlets within the muscle, it might be thought of as a second system in parallel with the actomyosin system, which, when it is made stiff, is capable of maintaining tension initially developed by the latter system.

JULY 14, 1959

The physiology of the predator-prey relationship existing between Paramecium aurelia and Didinium nasutum. JAMES D. EISEN.

A study of the population interactions existing in a three-level food chain was made to determine the effect of providing various monofloral cultures of bacteria to the prey, *Paramecium aurelia* Müller and the subsequent effect of the paramecia, when used as food, on the reproductive rate of the predator, *Didinium nasutum* Müller.

Pedigree *P. aurelia*, variety 4, stock 51:7, sensitives, were transferred from a non-living, non-bacterial medium to five monofloral cultures of Gram-negative, non-spore-forming bacteria, and to a wild bacterial culture. The ciliates grew well on the wild bacteria and on all the monoflorae except one. From this it was concluded that mixed bacterial cultures were not essential for the satisfactory growth of *Paramecium*, but that not all monoflorae were nutritionally adequate. The growth-inducing powers of the bacterial species were found to be variable.

Clonal didinia were excysted at the beginning of each of the 16 five-day isolation-culture replications, sterilized, and placed along with 300 washed paramecia of each line into individual depression slides. Division rates of the predator on each *Paramecium* line were recorded for the first three days of each replication, and the means compared statistically. *Didinium* preying upon wild-bacteria-fed paramecia manifested a division rate of $4.9557 \pm .0083$; on the five monofloral-fed paramecia, division rates were $.8510 \pm .0520$, $1.0942 \pm .0552$, $1.7721 \pm .0388$, $1.9700 \pm .0237$, $2.0527 \pm .0343$.

After several generations, all didinia except those on wild-bacteria-fed paramecia died. The statistically different division rates and subsequent death of the didinia appeared to be caused by nutritional deficiencies in the monofloral-fed-paramecia, possibly vitamins or amino acids, which were induced by the bacterial food ingested by the prey.

The inadequacy of paramecia fed on a monofloral culture of *Bacillus pyocyaneus* may have accounted for the death, rather than the encystment, of *Didinium* in Gause's classical predator-prey cycle. Gause's theory, that "immigrations" of both the predator and prey were necessary for the maintenance of the cycle, may not be wholly correct.

Life cycle and metabolism of a marine nematode, Enoplus communis. WOLFGANG WIESER.

The life history and metabolism of a marine nematode, *Enoplus communis* Bastian, was studied. The species has an annual life cycle, spawning taking place in early spring. Ma-

turity is reached in fall and early winter, and both sexes over-winter with their gonads fully developed.

The metabolism is not temperature-compensated and there is no difference in respiration between the two sexes.

The respiration rate is fairly low in the fertilized, uncleaved eggs (about 600 $mm.^3$ O_2/g./hr.). Right after hatching the oxygen consumption increases sharply to a value about three times that of the eggs. Then it drops quickly, but in animals weighing between 6 and 10 μg another sudden rise occurs, followed by a second drop and subsequent levelling of the curve. The first increase in metabolic rate from egg to first juvenile stage might be due to the activation of metabolically important substance. The following changes, however, are most likely correlated with the process of molting and are probably due to changes in the percentage of dry tissue matter in animals prior to and after ecdysis.

The large fluctuations in respiration rate occurring in animals of different size, despite a constant surface/volume ratio, suggest that body surface proper does not control oxygen consumption in this species. Rather, the relationship between size and metabolism has to be considered as a demonstration of the law of relative growth with respect to cells and cell constituents.

Uptake of strontium by Syracosphaera carterae. RALPH A. LEWIN.

Coccolithophores (Chrysophyta) frequently constitute the main component of marine phytoplankton, especially in warmer seas. Since their cells are characteristically invested in an armature of sculptured scales of calcium carbonate (coccoliths), they are evidently one of the chief biological mechanisms for calcium deposition in nature. The degree of strontium incorporation by coccolithophores is therefore of practical importance in relation to the rate of entry of Sr^{90} into the biosphere.

A synthetic sea-water medium was devised, containing 3.4 m*M* of Ca, pH 6.8. In replicate flasks, 0.0, 0.5, 1.0 and 2.0 m*M* of Sr were added. After autoclaving they were inoculated with a pure culture of *Syracosphaera carterae*, and were maintained at 22° C., aerated, and illuminated constantly at 2500 lux, for 25 days. The coccoliths were then harvested by differential centrifugation, washed, dried, and examined by x-ray diffraction (Dr. I. Fankuchen) and flame photometry (Dr. T. J. Chow).

In the absence of Sr, they consisted of pure calcite. In the presence of Sr, the incorporation or concentration factor (atoms Sr/Ca in coccoliths : Sr/Ca in medium) was approx. 0.02, indicating a high degree of discrimination against this element, under the defined experimental conditions. It is not known whether this selectivity is attributable entirely to chemical forces during crystallization of the calcite, or partly to biological specificity.

JULY 21, 1959

Motion pictures of mating behavior of Tetrahymena pyriformis rendered amicronucleate by x-ray treatments. CARL CASKEY SPEIDEL AND GEORGE GILBERT HOLZ, JR.

As previously reported by one of us (C. C. S.) for three strains of *Tetrahymena corlissi* the micronucleus, a genetic center rich in deoxyribonucleic acid, was eliminated by repeated severe x-ray treatments. Since mating in this species had not been observed, it became desirable to use another species in order to watch the mating behavior of amicronucleate tetrahymenae. This was done for axenic clonal cultures of *Tetrahymena pyriformis*, Mating Types I and II, Variety 1 (G. G. H.).

After receiving a cumulative dose of 2400 kr from four separate x-ray treatments of 600 kr each, the surviving progeny of Mating Type I consisted of amicronucleate individuals; those of Mating Type II consisted of both amicronucleate and micronucleate individuals. The mating behavior of the following combinations was observed and recorded by cinephotomicrography: (1) normal unirradiated tetrahymenae of Mating Types I and II, (2) x-rayed Types I and II, (3) normal Type I and x-rayed Type II, and (4) x-rayed Type

I and normal Type II. No difference was noted in the mating behavior of these four combinations. Conjugation of amicronucleate individuals of x-rayed Types I and II occurred. During conjugation of all four combinations, triples were occasionally formed. Descendants from a single mating pair of x-rayed Types I and II, kept in the same bacterized culture, still conjugated after six months. Likewise descendants from x-rayed Types I and II, kept in separate bacterized cultures for seven months, still conjugated. It is concluded that the presence of the micronucleus is not necessary for the mating reaction to occur.

This investigation was supported by a research grant (RG-4326 C2) to C. C. S. from the National Institutes of Health, Public Health Service.

Lysosomes and the physiology and pathology of cells. ALEX B. NOVIKOFF.

In 1956, with Beaufay and deDuve, we reported that electron microscopy revealed the presence in isolated lysosome fractions from rat liver of bodies resembling pericanalicular bodies seen *in situ*. However, the identification of the pericanalicular dense bodies as lysosomes was provisional since the fractions also contained a great many mitochondria. With Edward Essner, we have recently obtained support for this identification from an electron microscopic and cytochemical study of hepatocellular pigments in man. Lipofuscin, biliary pigment, iron, or the unknown pigment of chronic idiopathic jaundice may appear within the pericanalicular dense bodies, converting them into characteristic pigment granules. The granules retain acid phosphatase activity. Parallel electron microscopic and staining studies of liver, kidney, and macrophages show that the Gomori staining reaction for acid phosphatase activity, when performed on frozen sections (or smears) of cold formal-calcium-fixed tissues (or cells), is a reliable means of visualizing lysosomes. Other studies support deDuve's recent suggestion that lysosomes play important roles in pinocytosis, phagocytosis and lytic processes. Among the material studied are pinocytosis vacuoles of *Chaos chaos;* digestive vacuoles of *Ameba proteus;* phagocytosis in erythrophagocytes and in macrophages of spleen, liver, lung and other tissues of rat, mouse, and man; many normal tissues; rat kidney following intraperitoneal protein injection; cytolyzing cells in normal physiological or developmental circumstances (atretic ovarian follicles, resorbing tadpole tails, regressing chick Mullerian ducts); dying cells, as in keratinization of squamous epithelia, and pathological situations (following ligation of vasculature to liver or kidney, or of bile duct, or of ureter; or during feeding of carcinogenic azo dyes.) From our survey we conclude that most bodies described by electron microscopists as "microbodies," "cytosomes," and "large granules" are lysosomes—cytoplasmic organelles delimited by "single" outer membranes and possessing high levels of acid phosphatase and other hydrolases with acid pH optima.

JULY 28, 1959

On the occurrence of myoglobin and cytochrome oxidase activity in the cartilaginous odontophore of Busycon canaliculatum. PHILIP PERSON, JAMES W. LASH AND ALBERT S. FINE.

Both myoglobin and cytochrome oxidase activity have been identified in the above tissue. Aqueous solutions of the oxidized myoglobin possess α, β and γ peaks at 574–575, 538–539 and 415–416 mμ, respectively. In the dithionite reduced state, the α and β peaks are replaced by a lower, single broad hump, between 540–565 mμ. The soret absorption decreases and moves to 430 mμ. The pyridine hemochrome prepared from the myoglobin has an α peak at 554–555 mμ, a β peak at 524–525 mμ, and a third, smaller peak at 480 mμ, in the visible spectrum. The soret absorption of the hemochrome is at 417–418 mμ. Washed particulates prepared from homogenates of the tissue could oxidize reduced cytochrome *c*, as demonstrated in spectrophotometric experiments. The loss in absorbance of reduced cytochrome *c* at 550 mμ was $-0.264/3$ minute interval/mgm. dry weight. In a manometric assay for cytochrome oxidase activity using hydroquinone as substrate and added cytochrome *c*, the Q_{O_2} (dry weight) was 9. This is the first reported occurrence of myoglobin and cytochrome oxidase activity, together, in a cartilage type tissue.

Electron microscope study of the sperm of Crassostrea virginica. PAUL S. GALTSOFF AND D. E. PHILPOTT.

Mature spermatozoon of the oyster examined under highest magnification available with phase contrast microscope was observed to contain a light refracting body at the center of the nucleus under the acrosome. This observation led to a detailed study of the sperm by means of electron microscopy. Sperm balls removed from the tubules of the testis were fixed in buffered 1% osmic acid, embedded in plastic, and sectioned. The structure of the spermatozoon was reconstructed from over 300 photographs taken at various magnifications and at different planes. The tip of the slightly granular and homogeneous nucleus is covered by a conical acrosome of highly osmiophilic substance. The center of the nucleus is occupied by an egg-shaped body which extends from the apex of the acrosome almost to the base of the nucleus. This structure is undoubtedly a permanent feature of the spermatozoon and is probably related to the formation of the axial filament. It has been named by us the "axial body." The core of the axial body consists of a central stem of slightly denser material.

The centriole, at the base of the nucleus, is a hollow, cylindrical body measuring about 0.2 μ in length and 0.1–0.18 μ in width. The centriole walls are constricted into several sections of a material of alternating density. Nine bands of denser material are clearly seen at the cross-section of the centriole. The lower part of the centriole is in close contact but separated from the basal plate of the tail. Four mitochondrial bodies surround the centriole and are connected to its wall. The structure of the tail is similar to that of a cilium; it consists of a pair of axial filaments surrounded by a ring of nine double filaments emerging from the basal plate. The pair of axial filaments does not originate from the basal plate and consequently the proximal part of the tail consists only of nine peripheral filaments. The peripheral filaments of the tail are connected to the outer walls and to the axial filaments by radial trabeculae.

AUGUST 4, 1959

Some structures found in electron microscopic studies of an amphibian tumour. VINCENZO LEONE.

In the course of an electron microscopic study of a tumour (reticulo-lympho-sarcoma) induced by injecting methylcholanthrene into the newt, *Triturus cristatus* L., unusual structures were found in some of the cells, in the spleen.

These studies have been carried out in the Dept. of Zoology, University of Milano, Italy, using a Philips 75 KV electron microscope, and at the Institute du Cancer Gustave Roussy, Villejuif, France, using a Siemans electron microscope: Elmiskop I. Large areas of the cytoplasm containing masses of dense material, bounded by a limiting membrane and containing granules within vacuoles, were seen between the more or less altered organelles of the cytoplasm. Similar masses containing vesicles and large granules were likewise observed. The latter were usually more dense, and showed an ill-defined internal structure. It is possible that the two types of formations described are similar reaction bodies in different stages of evolution. In some cases, cells in both the spleen and the liver were crowded with bacteria and they showed a typical reaction; the cytoplasm contained large, round dense bodies surrounded by membranes. At times they contained vacuoles of various sizes and shapes. Although in most cases the cytoplasmic reaction masses surround and engulf bacteria, these masses are not always related to the bacterial bodies. Frequently the reaction phenomenon is represented by a clearly visible series of concentric membranes. The bacterial bodies within the masses give the appearance of being somewhat digested. Osmiophilic bodies and (empty?) vacuoles are also frequently found. In some cases numerous bacterial bodies, surrounded by one or two membranes, are found in the reaction zones; in others only one or two bacteria are found within the reaction zone.

I would like to emphasize that this amphibian material illustrates a very spectacular reaction to a living (bacterial) agent. This agent produces a very pronounced but not a lethal influence. The observed association of cell (macrophage) + bacteria (non-pathogenic) gives a clearer picture of a process (phagocytosis?) than is the case with most of the agents usually considered as responsible for the induction of inclusion and reaction bodies.

Electron microscope study of the integument, flame cells and gut of the human parasite, Schistosoma mansoni. ALFRED W. SENFT.

An electron microscope study of the trematode, *Schistosoma mansoni*, was undertaken in order to demonstrate the basic structure of certain portions of this blood parasite. It was hoped that clues concerning the physiology of the integument, flame cell structures, and gut might be deduced from the ultrastructure morphology.

The integument was found to consist of a multivacuolated, highly frondular surface, penetrated at intervals by spines. The presence of an enormous number of "Swiss cheese" lacunae in the outermost layer suggests that the integument is a huge absorbing surface which transports the major portion of the nutrients required by the schistosome. A thin osmophobic membrane is immediately subjacent to the outer surface. The spines appear to be imbedded in this clear membrane. Muscle layers, both horizontal and circular in arrangement, were seen beneath the osmophobic layer. Small canaliculi proceeding from deeper body areas and penetrating the integument were suggested in some views, although one could not be certain that these were not artifacts.

The flame cells were found to consist of bundles of about 80 cilia. These are enclosed by a single or double layered membrane. The membrane itself has a series of stiffening members which are arranged in a stockade fashion around the bundle of cilia. It is postulated that this membrane is freely permeable to body fluids. The stiffening members probably keep the lumen of the flame cell patent in the presence of variable pressures.

Cilia were noted to have two or four central fibrils, and nine pairs of fibrils arranged concentrically around the center fibrils. Distal to the flame cells, collecting tubules were shown to have lamellated membranes. These might function as check valves, preventing reverse flow of fluid which the flame cells put in motion.

The gut was shown to have an extensive fibrillar or microvillar surface.

Fine structure of skeletal muscle from Limulus polyphemus. G. W. DE VILLAFRANCA AND D. E. PHILPOTT.

Observations of *Limulus* skeletal muscle with both phase contrast and electron microscopes reveal sarcomeres, about 7.5 μ at rest length, which are cross-striated. The dark A band (5.0 μ) is of uniform density: no H or M bands are present. The I band (2.5 μ) is bisected by a dark Z band which, in some sections, seems to be continuous across the whole fiber and terminates on the sarcolemma in a plate-like structure. There is, however, complete separation by a membrane of the sarcolemma and Z band. In other sections the Z band is interrupted by vesicular material between two mitochondria. Mitochondria are relatively rare and occur on both sides of the Z band region, adjacent to the I band. Extensive vesicular material, "sarcoplasmic reticulum," occurs at the level of the A band, encircling the myofibrils, and connecting with the cell surface at the Z band level.

The A band consists of large filaments about 140 Å in diameter and spaced from 170 to 200 Å apart. These filaments run continuously through the A band, parallel to the major axis, and are of constant diameter. Apparently the large filaments are interconnected by small (30 Å), filamentous "cross-bridges" which, in some cases, appear to be coiled around the large filaments. In electron micrographs of very thin sections (about 1 filament thick) no "secondary" filaments are observed between the large "primary" filaments; nor are any observed in sections where large filaments run in and out of the plane of section. In cross-section, one may observe an hexagonal pattern of large filaments with cross-bridging filaments between and at right angles to the large filaments.

On the basis of these observations, horseshoe crab muscle could not contract by a sliding filament mechanism as proposed for other muscle by Huxley, but, as in vertebrate smooth muscle, must contain a contractile system within a single set of filaments.

AUGUST 11, 1959

Present status of the problem of plasmalogen structure. MAURICE M. RAPPORT.

Plasmalogens are glycerophosphatides that release higher fatty aldehydes under hydrolytic conditions. The organic radical responsible for this reaction was recently shown to be an

α, β-unsaturated ether. It is unique among natural products: carbon in the aldehyde oxidation state participates in double bond formation (hydration of this bond, followed by hydrolysis, generates aldehyde). This double bond, much more reactive than the normal type, gives rise to several remarkable addition reactions.

Pure native plasmalogens are not yet obtainable. However, a pure, crystalline derivative, known for many years, has been assigned an acetal structure. This was shown to be incorrect, and the true α, β-unsaturated ether structure was first conclusively proved, using crystalline preparations from both ox muscle and brain. This proof was based on the demonstration that hydrogen addition rather than hydrogenolysis produced the loss of aldehydic reactions attending hydrogenation. Confirmation was obtained by means of a reaction specific for the "activated" unsaturation in α, β-unsaturated (vinyl or enol) ethers: addition of iodine in aqueous methanol. Under conditions where such ethers add 95% of the stoichiometric quantity of iodine, no reaction is given by ordinary double bonds, aldehydes, acetals, hemiacetals, or acetylenes.

Availability of two independent, stoichiometric, analytical methods presented a rare opportunity for examining the total plasmalogen content of different tissues. Thus, the correlation of iodine uptake and aldehydogenic content (based on p-nitrophenyl-hydrazone formation) for total lipid extracts of tissues from rabbit, rat, and man showed that almost all mammalian plasmalogens must be α, β-unsaturated ethers.

Unsolved structural problems are three: 1) is the aldehydogenic chain connected to the primary or secondary hydroxyl group of glycerol (the remaining hydroxyl group is esterified to fatty acid); 2) are some plasmalogens not phosphatides (reported for Asterias); and 3) do true acetal plasmalogens occur naturally (reported for Anthopleura)?

The incorporation of radioactive label into RNA: synthesis or terminal addition. W. S. Vincent and Elyane Baltus.

An analysis of the specific activities of individual nucleotides from the RNA of starfish oocyte cytoplasm after exposure to P^{32} has revealed striking inequalities in the labelling of specific nucleotides. Such inequalities suggested that terminal addition of nucleotides to a pre-existing RNA molecule was taking place, rather than synthesis of new higher polynucleotides.

At six hours the relative specific activity of nucleotides isolated from KOH hydrolysates was Cy: 41; Ad: 42; Gu: 10; Ur: 6. Such data would be consistent with the hypothesis that two cytidylic acid molecules were added to an RNA molecule terminating with an adenylic acid residue. Partial confirmation of this hypothesis is found in shorter time experiments, where after two-hour incubation the following relative specific activities were determined: Cy: 20; Ad: 60; Gu: 14; Ur: 6. Analysis of a diesterase digest revealed the following distribution of label: Cy: 46; Ad: 1; Gu: 0; Ur: 53.

On the basis of these data it is likely that most of the radioactive label incorporated into the starfish cytoplasm RNA occurs as the addition of two cytidylic and a terminal uridylic acid residue to an adenylic acid residue present on a pre-existing RNA molecule.

On the basis of the experiments cited, we conclude that a precise analysis of the distribution of label in the RNA molecule is necessary in order to determine whether or not net synthesis has occurred. Results from autoradiographic techniques become particularly difficult to assess, for they are not susceptible to rigid biochemical control.

August 18, 1959

The action of glycerol on protoplasm. L. V. Heilbrunn.

Various authors have shown that when some types of cells are previously treated with 10–30% solutions of glycerol, these glycerinated cells become extremely resistant to very low temperatures. In muscle physiology, treatment of muscle fibers with 50% glycerol has become very common practice. And yet there has been but little effort to determine what the glycerol does to the physical state of the cells which have been bathed in it.

Unfertilized eggs of Arbacia and Chaetopterus and fertilized eggs of the clam Spisula were exposed to 5%, 10%, 15% and 20% glycerol in sea water and the viscosity of the

cytoplasm was then determined by the centrifuge method. Even in the 5% solution there is a large increase in viscosity; higher concentrations cause an increase in viscosity so great that it can no longer be measured. Muscle fibers were exposed to 50% glycerol and were then injected with isotonic KCl solution. If such a solution is injected into fresh muscle fibers, it spreads rapidly through the fiber—also, when the micropipette is removed there is no trace of where the pipette had been. However, in the glycerinated fibers the injected fluid remains as a discrete droplet; and when the micropipette is removed, the hole it had caused in the fiber remains clearly evident. It is as though a sharp instrument had been plunged into a piece of cheese.

Obviously, both in marine eggs and in muscle fibers, glycerol can cause a gelation of the protoplasm. Thus one can understand the great resistance of some glycerinated cells to low temperatures; for ice crystals, which can readily form in protein sols, can not form in gels. Also, in the case of muscle, contraction of a glycerinated muscle fiber involves some change in a gel, whereas the normal muscle fiber is largely fluid.

Aided by a grant from the National Science Foundation.

Electrophoretic studies on protoplasm. WALTER L. WILSON AND K. S. SWAMI.

Observations were made on unfertilized marine eggs subjected to an electrical current to determine whether the cytoplasmic inclusions move to the cathode, in which case they bear a positive charge, or to the anode, in which case they bear a negative charge. The eggs of Arbacia, Asterias, and Echinarachnius in sea water undergo cytolysis when subjected to an electrical current. This reaction does not occur in the absence of calcium ion. Thus, eggs in sodium citrate (0.35 *M*) do not undergo cytolysis during the flow of an electrical current. Under these conditions granules move toward the cathode and in almost all cases in Arbacia and Asterias some granules move toward the anode, leaving a clear area on the cathodal side of the egg. However, the clear area on the cathodal side of the egg is much smaller than that on the anodal side. In the egg of Chaetopterus in sea water there is a breakdown of cortical granules and a movement of granules toward the cathode, but if the current intensity is increased the whole mass of protoplasm seems to coagulate and moves toward the anode. The egg of Hydroides in sea water, or in sea water plus citrate, reacts like the eggs of Arbacia and Asterias in citrate. In the egg of Spisula in sea water there is a movement of granules and nucleus toward the cathode, but only after marked swelling of the egg. If these eggs are immersed in citrate for short periods there is a movement of granules toward the anode, but after about an hour the granules move toward the cathode.

This work was supported by a grant from the National Science Foundation, administered by Walter L. Wilson.

Germinal vesicle breakdown in the eggs of Spisula and Hydroides. FRANCIS T. ASHTON.

Germinal vesicle breakdown, an easily observable example of the first step in typical cell division, can be induced experimentally by many chemical and physical agents. These agents act either on the cortex, where they release calcium, which activates a protease system, or directly on the nuclear membrane. Allen (1953) showed that many agents, among them excess isotonic potassium and hypertonic solutions, act on the *Spisula* egg only in the presence of calcium. In addition, I have found that chymotrypsin will act in the absence of calcium. *Hydroides* eggs may be activated in the above ways, by heat and cold, and by trypsin in the absence of calcium.

If, on activation, calcium releases a protease, then enzyme inhibitors as well as calcium-binding agents should affect activation. Soy bean (trypsin) inhibitor and Ovomucoid delay but do not prevent germinal vesicle breakdown in *Spisula*. Iodoacetamide delays the breakdown and markedly reduces the number of eggs activated.

If *Spisula* eggs are homogenized in sea water and the homogenate centrifuged, the supernatant contains a substance which will activate fresh *Spisula* eggs. This activity disappears on the addition of soy bean inhibitor, and is reduced by heating. Homogenates made in citrate do not show the activity.

When *Spisula* eggs are activated with excess KCl in sea water and homogenized in citrated sea water at the time of nuclear breakdown, the supernatant contains an activating substance. The activity disappears on the addition of Ovomucoid or iodoacetamide. The peak of activity occurs at the time the germinal vesicles are breaking down, not earlier or later. The substance extracted from the homogenates is undoubtedly a calcium-activated protease, requiring SH groups. It is also the enzyme responsible for the breaking down of the germinal vesicle.

Aided by a grant from the National Science Foundation to L. V. Heilbrunn.

ELECTROBIOLOGY SEMINARS

JULY 20, 1959

The fine structure of synapses. A. J. DE LORENZO.

Examination of numerous synaptic junctions with the electron microscope has revealed a remarkable uniformity in fine structure, *i.e.*, most synapses are characterized by (a) closely applied limiting membranes of the synaptic processes separated by a cleft about 200 to 300 Å wide, with occasional areas of increased density; (b) presynaptic terminals containing mitochondria and clusters of "synaptic vesicles."

Utilizing this fine-structure model, smooth muscle of the iris and small intestine were examined with particular reference to innervation. Nerve endings in iris muscle are numerous and contain large numbers of vesicles. Synaptic membranes are separated by a cleft 75 to 100 Å wide. Individual muscle cells contain little endoplasmic reticulum and are separated by large intercellular spaces. On the other hand, intestinal muscle cells contain a paucity of recognizable nerve endings. Synaptic clefts are 75 Å wide and endoplasmic reticulum is more abundant in the muscle cells.

Since it has been suggested that "synaptic vesicles" which occur usually in presynaptic processes may be involved in chemical transmission, certain electrical synapses in the crayfish were also studied. Examination of the crayfish lateral giant-to-motor synapse reveals motor fiber processes terminating on the lateral giant pre-fiber in very close contiguity. A synaptic cleft about 75 Å wide separates the synaptic membranes. Curiously, only the *postsynaptic* processes contain clusters of vesicles and the presynaptic fiber demonstrates no structural specialization. Cholinesterase staining of this synapse was routinely negative.

These observations suggest that fine-structure differences exist at smooth muscle endings and in electrical synapses of the crayfish when compared with the classical terminal bouton. It would seem wise, however, to approach with caution any interpretation of the nature of synapses based upon dimensions of synaptic clefts and locations of "synaptic vesicles," until more evidence is forthcoming. Possible role of "synaptic vesicles" at electrical synapses remains unresolved.

Presynaptic activity in drug-induced neuromuscular facilitation. A. S. KUPERMAN, G. WERNER AND E. W. GILL.

Almost 60 compounds were tested for their capacity to facilitate neuromuscular transmission in the cat, and those found effective are divisible into two groups. In one group the dose-response regression lines for twitch potentiation are parallel and steep, and significant correlation exists between facilitating potency and anti-ChE activity. In the other group dose-response lines are parallel but flat, and there is no relationship to ChE inhibition. The structural requisites for twitch potentiation and curare antagonism are also clearly different for each group. Although this evidence indicates a functional distinction between the two classes, other evidence reveals a common presynaptic mode of action for all facilitating drugs. All agents produce post-activation repetitive discharge of motor nerve which originates from the axon terminals. Furthermore, this nerve terminal action, twitch potentiation, and curare antagonism are enhanced by tetanic nerve stimulation, double volleys, and subfacilitating doses of physostigmine; these effects are suppressed by anesthetic doses of pentobarbital and subparalytic doses of curare. Each of these modifications of drug-induced facilitation was shown to act exclusively through a presynaptic mechanism.

Accordingly, it is proposed that all facilitating agents have a common presynaptic action; superimposed on this, in consequence of a specific molecular configuration, is another action which bears relation to anti-ChE activity. In these latter molecules, the contribution of presynaptic and anti-ChE effects to facilitation cannot be separately evaluated. Therefore, since

these agents have a nerve terminal action which is greater than that induced by agents having only the presynaptic effect, the anti-ChE correlation may require explanation within a context other than the ACh-ChE theory of transmission.

The presynaptic events capable of being altered by drugs were found to be in the nature of nerve after-potentials. Recognition of these neuronal events which precede the transmitter process and are susceptible to distinct influences by chemical agents is necessary to the proper interpretation of pharmacologic studies; its significance to the physiology of mammalian neuromuscular transmission remains to be evaluated.

JULY 27, 1959

The chemistry of ion transport and its relation to excitation in nerve and muscle. BENJAMIN LOWENHAUPT.

Consideration of the answers to two questions has suggested a possible molecular model for excitation:

I. What may be known about the chemistry of ion transport?

A molecular model for transport, initially developed to explain transport in aquatic plants, is compatible with many biological materials. Its features include the following:

1. There are carrier molecules which have more or less specific chemical affinity for the transported ion and which successively bind ions from the ambient solution and release them back into solution.
2. The reactions for binding and releasing the ions are redox reactions of the carriers, *i.e.*, they constitute a redox pump.

II. Do chemical reactions for transport also occur for excitation and, if so, can insight into the chemistry of excitation be drawn from what is understood about the chemistry of transport?

Affirmative answers to this question indicate that excitation is a sudden oxidation of carrier molecules with concomitant release of ions, a wave of oxidation propagated along the cell. The name, *oxidation-wave hypothesis,* refers to this molecular model of excitation. It is supported by the following arguments:

1. A redox pump occurs in excitable cells, as in cells and tissues which transport. Furthermore, its activity parallels the rate of the bioelectric changes of excitation.
2. The kinetics of excitation appear the same as the kinetics of reactions for binding and releasing ions, the same reactions as for transport.
3. A sudden oxidation does occur during excitation.
4. If oxidizing muscle during excitation causes it to contract, the coupling between muscle excitation and contraction is established. Experimental observation is that oxidized muscle does contract, whereas it relaxes when reduced.

AUGUST 3, 1959

Effects of estrogen and progesterone on single uterine muscle fibers in the rat. J. M. MARSHALL.

White rats were ovariectomized and treated as follows: group 1, 6.0 μg. estradiol benzoate daily for five days; group 2, 6.0 μg. estradiol for three days, then 1.6 μg. estradiol plus 12 mg. progesterone for five days; group 3, untreated animals. Membrane potentials were recorded from single uterine fibers, and the tension was recorded from the entire uterine horn.

The untreated uterine fibers were quiescent, having a mean resting potential of 35.2 mv. Fibers from the estrogen-dominated uteri were rhythmically contractile and had a mean resting potential of 57.6 mv. A train of action potentials accompanied and preceded each contraction of the muscle. In certain areas the fibers showed pacemaker-like characteristics, *i.e.*, slow membrane depolarization between action potentials. Progesterone-dominated fibers had significantly higher resting potentials, mean 63.8 mv., but no localized pacemaker areas. Action potentials did not consistently precede or accompany contractions.

In groups 1 and 2, acetylcholine stimulated contractions, lowered the membrane potential and increased the discharge rate of action potentials. Epinephrine diminished contractions, raised the membrane potential and abolished the action potential discharge. The untreated uteri were unresponsive to both of these substances.

GENERAL SCIENTIFIC MEETINGS

AUGUST 24–27, 1959

Abstracts in this section (including those of Lalor Fellowship reports) are arranged *alphabetically by authors* under the headings "Papers Read," "Papers Read by Title," and "Lalor Fellowship Reports." Author and subject references will also be found in the regular volume index.

PAPERS READ

Contraction of muscle induced by direct light stimulation. PHILIP B. ARMSTRONG.

It has long been known that contraction can be induced by light stimulation in certain muscles without the mediation of nerves. The classic examples are the sphincter pupillae muscles of some of the fishes and many of the amphibians. Among the fishes the sphincter pupillae of the eel serves as a particularly good preparation for experimental study.

After excision of the eye the cornea is dissected off, the posterior three-fourths of the eyeball and the lens removed, and the remaining part of the eye, which includes the iris, is pinned out in Ringer's solution on beeswax with the outer surface up. The preparation is mounted on the stage of the microscope, a substage lamp is used and changes in pupillary diameter are measured with a micrometer in the ocular of the microscope. Light from the substage lamp does not reach the sphincter pupilla because of the heavy melanin pigment on the posterior surface of the iris.

If the preparation is placed in the dark, adaptation takes place with the pupil becoming fully dilated within 20 minutes. If light is then thrown from above on what is the front of the iris, constriction follows which comes on after a short refractory period and is complete within 30 seconds with light of adequate intensity. Graded responses are obtained with properly selected intensities of light. Adaptation of the sphincter pupillae to light is striking. If the pupil is maximally constricted by light and the illumination of the iris continued at this intensity, adaptation takes place with a dilation of the pupil to about 35% of the initial maximal constriction in 35–40 minutes. With submaximal light constriction adaptation also takes place. However, increased light intensity will induce constriction from the light adapted condition.

Supported in part by NIH Grant B-643.

Inhibition of fertilization and agglutination in Arbacia by an extract of Fucus. JOSEPH M. BRANHAM AND CHARLES B. METZ.

Investigations in Runnström's laboratory have shown that certain extracts of the brown alga *Fucus vesiculosus* inhibit fertilization in sea urchins. It seemed of interest to examine the local material for similar effect and to investigate further the mode of action of the inhibitor(s).

Extracts which inhibited fertilization in Arbacia were obtained by repeated extraction of fresh *Fucus vesiculosus* in distilled water at room temperature. The combined extracts were concentrated under vacuum at 50° C. and ten volumes of 95% ethanol added. A precipitate was discarded and the ethanol distilled from the soluble fraction under vacuum. This concentrated solution was finally dialyzed against sea water. Such solutions inhibited fertilization in dilutions exceeding 6×10^{-3}. Fertilization inhibition did not result from an irreversible action on the sperm, for sperm washed from the extracts showed undiminished fertilizing capacity. The *Fucus* extract appears to inhibit eggs irreversibly, for washing did not restore

fertilizability to treated eggs. However, even following prolonged treatment in the extracts fertilized eggs were capable of some development.

The *Fucus* extract not only inhibited fertilization but also the agglutination of sperm by fertilizin. This action did not result from an irreversible interaction of the extracts with fertilizin since treatment with activated charcoal restored agglutinating action to non-agglutinating inhibitor-fertilizin mixtures. The agglutination inhibiting action appeared to result from a blocking of the surface of the sperm. Sperm washed from the extracts were not agglutinated by fertilizin. Moreover, the inhibiting effect was reduced or removed from the extract by sperm. Furthermore, sperm treated with the *Fucus* extract did not reduce the agglutinating power of fertilizin as did control sperm. Thus it seems that the *Fucus* extract reacts irreversibly with the antifertilizin on the sperm to render it unreactive to fertilizin. However, such blocking does not diminish the fertilizing capacity of the sperm.

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Central nervous aspects of firefly excitation. JAMES F. CASE AND JOHN BUCK.

We previously reported the occurrence, in the posterior abdominal nerve cord, of characteristic volleys of nerve impulses which precede each voluntary flash. Subsequent volleys have now been recorded from the subcuticular surface of the lantern and have been shown to precede induced as well as spontaneous flashes. Although double volleys initiate double flashes, and volleys recorded from widely separated regions of the lantern may differ slightly, there is little apparent correlation between volley duration and impulse frequency and the intensity of the succeeding flash or area of photogenic tissue involved. This suggests either that there is further, as yet undiscovered, neural modulation or that the tissue has considerable effector lability. The possibility of secondary modulation is perhaps supported by the fact that although flashes are always preceded by volleys, not all volleys eventuate in flashes. Furthermore, some individual fireflies are quite refractory to electrical stimulation, or even to the induction of pseudoflashes after hypoxia, until aroused. This inactive state may be associated with the persistent endogenous diurnal periodicity of spontaneous flashing activity described some years ago. Second order excitation could involve either a separate, more profuse peripheral nervous network, or a state of subthreshold facilitation maintained by "trophic" central activity correlated with behavioral state. There is considerable neural activity between volleys recorded from the cord, and some single spikes are seen between volleys in the lantern, but seemingly not enough to account for maintained priming. However, additional indications of central involvement are seen in the facts (a) that both voluntary and induced flashes may be completely inhibited by optic nerve stimulation, and (b) that eserine induces, in lanterns with central connections but not in denervated ones, asynchronous unit effector activity which may go through regular cycles of block and recovery.

Purification and properties of a ribonuclease from squid. MARY EDMONDS AND JAY S. ROTH.

The isolation and comparative study of ribonucleases differing from bovine pancreatic ribonuclease is of value for determining the relation of protein structure to enzyme activity, since the structure of the bovine enzyme is well known. Ribonucleases of known specificity are also useful for investigations of polynucleotide structure.

Squid gill and caecal fluid were examined. The former contains modest amounts of an acid ribonuclease associated with a particulate fraction that sediments at $10{,}000 \times g$. Caecal fluid contains large amounts of a soluble acid enzyme and for this reason was studied extensively. The caecal enzyme has an optimum pH at 5.3, is not affected by sulfhydryl reagents and is stable to heating at 60° C. for 5 minutes. It precipitates at 80% saturation with ammonium sulfate and is stable to treatment with acid. Thus, except for the pH optimum, it resembles crystalline bovine pancreatic ribonuclease in some respects. The squid caecal enzyme was purified approximately 300-fold by heat treatment, absorption of impurities by DEAE cellulose and, finally, salt gradient elution from a carboxymethyl cellulose column. The purified ribonuclease was free of phosphodiesterase and phosphatase activity. It split both

polyadenylic and polyuridylic acids but did not appear to hydrolyze cyclic uridylic-, cyclic cytidylic-, or cyclic adenylic-3′,5′-phosphates appreciably. It also did not hydrolyze adenosyl-3′- or 5′-benzyl phosphates and, therefore, may require a purine-purine, purine-pyrimidine or pyrimidine-pyrimidine diester phosphate linkage for activity.

(Supported by grants from the National Cancer Institute and National Science Foundation.)

Isolation and characterization of cortical granule-hyaline material of the Arbacia egg. R. G. FAUST, R. F. JONES AND A. K. PARPART.

The material released from the cortical granules of the egg of *Arbacia punctulata* upon fertilization appears to be a mucoprotein. It has been isolated by allowing de-jellied, unfertilized *Arbacia* eggs to settle from sea water suspended above a 20–80 mixture of isosmotic sucrose and glycerol. During the settling the cortical granules explode and release their contents which is soluble in the non-electrolyte. Light centrifugation, removal of the supernatant sea water and collection of the egg-free sucrose-glycerol mixture gives a solution which, when brought to pH 2 to 3 with HCl, forms a white precipitate. This precipitate is washed 4 times with H_2O-HCl at pH 2–3, packed centrifugally and frozen dried.

The mucoprotein thus obtained contains 11% nitrogen, *ca.* 2% hexoseamine, 28% polysaccharide and 70% protein. Glucosamine and galactosamine are the amines, while galactose, fucose and mannose are present in descending concentration. No ribose is observable. The protein contains 17 amino acids. Analytical centrifuge data indicate a single molecular species of about 100,000 M.W.

This cortical granule mucoprotein is digested by α-amylase, but not affected by β-amylase, hyaluronidase, lipase, trypsin or papain (at pH 6); nor is it affected by pepsin (at pH 2). In all of these respects it resembles the hyaline layer of the fertilized egg. Both the cortical granule mucoprotein and the hyaline are depolymerized in the presence of non-electrolyte and both are repolymerized when Ca or Mg are added. Both show marked contraction at pH 2 to 3 and reversal at pH 7 to 8.

Cation exchange in the erythrocytes of the mackerel, Scomber scombrus. JAMES W. GREEN, GEORGE HOLSTEN AND T. A. BORGESE.

Red cells of fresh mackerel blood, obtained from pooled samples from a number of fish by heart puncture, were washed twice in 0.26 *M* NaCl and incubated at 15° or 25° C. in this medium in 10–20% cell suspensions in the presence of trace amounts of Na^{24} or K^{42} and 5–10 mM/l. of glucose. Influx or efflux of Na or K was measured over a two-hour period during which the cell concentrations of Na and K remained essentially stable at a level of 77.3 and 130.2 mM/l. cells, respectively. In the absence of information to the contrary the ions were treated as being freely exchangeable and their movements as occurring in a steady-state, two-compartment system. K flux was found to average 20.2 and Na, 50.9 mM/l. cells/hr. at 25° C. Some variability in the exchange rates from experiment to experiment was found. These exchange rates are higher than have been reported for man, beef or duck red cells and are higher than human red cells even when a comparison is made between the exchange rates per unit surface area of red cell. These measurements are interpreted to mean that the penetration rate of Na and K in mackerel red cells is greater than similar rates previously reported for other species, and indicate either a higher metabolic rate or the use of a larger fraction of metabolic energy for the maintenance of ionic equilibria than is found in these other species.

Movements of the cell membrane of some fresh- and salt-water amebae. J. L. GRIFFIN AND R. D. ALLEN.

The behavior of the cell membrane of amebae during locomotion has been an important consideration in some theories of ameboid movement. The surface tension theory became untenable when Jennings showed that the membrane moved forward as the ameba moved and not backward as the theory demanded. It has been assumed in some more recent considerations of ameboid movement (Goldacre and Lorch) that the membrane is formed at the front of the

cell and destroyed at the rear. This assumption is not supported by the evidence reported in the past by Jennings, Dellinger, Schaeffer, and especially Mast, who observed the behavior of various inert particles attached to the surface of moving cells.

We have made further observations on the behavior of such inert particles (carmine, Chinese ink) attached to the surface of *Amoeba proteus, A. dubia, A. discoides, Chaos chaos, Pelomyxa palustris,* and several small species of marine and fresh-water amebae. In amebae exhibiting "walking movements" and in those attached near the tail, particles attached to the plasmalemma move forward with the ameba. In amebae which are firmly attached to the substratum at the front, the membrane and attached particles roll forward over the ameba as the specimen advances. The plasmalemma is not formed and destroyed every time the organism moves its own length. Instead, as Mast showed, the cell membrane slides freely over the hyaline ectoplasm except at those places where the ameba is attached to the substratum, and perhaps in the tail region. The ameba can, however, gradually increase its surface area in order to replace plasmalemma consumed in the formation of food cups or to allow for marked changes in form.

The block to cell division produced by deuterium oxide. PAUL R. GROSS, WILLIAM SPINDEL AND DELBERT E. PHILPOTT.

Renewed interest in the effects of D_2O on biological systems has stimulated recent studies on a variety of organisms. Although showing marked systemic toxicity in mammals, heavy water appears to have a more direct and severe effect upon rapidly proliferating cells and tissues. The present studies were undertaken in order to examine the D_2O effect on mitosis in systems where cell growth is not a source of experimental confusion. In *Arbacia* eggs, fertilization occurs normally in sea waters containing up to 25 volumes per cent D_2O. At higher concentrations, the number fertilized declines sharply, so that in 96% D_2O, only 2% of the eggs are fertilized whilst control cells in reconstituted normal water show 90–100% fertilization. The effect of D_2O on mitosis and cytokinesis is even more severe. In media containing more than 75 volumes per cent D_2O, mitosis is blocked at all stages; cells with cleavage furrows are arrested in the condition obtaining at the time of immersion. At 50% D_2O, many cells remain arrested; those not blocked suffer cleavage delays of three to four times the normal interval. Lower concentrations produce progressively smaller effects, and below 10% D_2O, the early cleavages proceed normally. The D_2O effect is at least partially reversible, even when the cells have been immersed in pure D_2O-sea water for more than an hour. Washing in sea water permits a significant number of cells to escape the block, and the resulting cleavages are usually irregular and multiple, so that if residence in D_2O has not been too prolonged, the treated eggs tend to "catch up" with the controls. Essentially identical findings are obtained with eggs of *Chaetopterus.* Electron microscope studies show the treated cells to have suffered multiple, localized gelations in the peripheral cytoplasm.

The effects of sperm on S^{35}-labelled Arbacia fertilizin. RALPH R. HATHAWAY.

The production of S^{35}-labelled *Arbacia* fertilizin has permitted observations of the fate of sulphur constituents of fertilizin during reactions with sperm. Previously reported experiments showed that, at times, the radioactivity was adsorbed to sperm, but that other conditions resulted in little or no association of S^{35} activity with sperm after mixing with labelled fertilizin. These results were interpreted as demonstrating a two-step reaction between sperm and fertilizin: (1) adsorption of fertilizin to sperm, (2) release from the sperm of part or all of the fertilizin molecule. These results have been confirmed and clarified in an experiment which permits observation of uptake and release of fertilizin-bound isotope in a single reaction mixture. Sperm and labelled fertilizin were mixed and centrifuged within 30 seconds. The supernatant (No. 1) was sampled for radioactivity. The sperm were re-suspended in the same supernatant and left at room temperature for 45 minutes, and then re-centrifuged. This supernatant (No. 2) was sampled. In 10 experiments it was found that supernatant No. 1 contained $28.0 \pm 3.6\%$ of the total radioactivity, while supernatant No. 2 contained $69.1 \pm 10.0\%$. Therefore, in the early seconds of the reaction, the sperm adsorbed about 72% of the labelled material, and in 45 minutes these same sperm released all but about 31% of the radioactivity.

The fraction 31/72 represents a retention by the sperm of 42% of the radioactivity initially adsorbed. Correspondingly, 58% of the initially adsorbed material is released within ¾ hour.

Labelled material released by sperm was found in dialysis to be non-diffusible. Released substances are not appreciably adsorbed to fresh sperm when treated a second time. Similarly, reacted sperm were completely unable to adsorb fresh labelled fertilizin.

Heterologous sperm (*Spisula, Pecten, Chaetopterus*) adsorb fertilizin slower than *Arbacia* sperm, and do not release any labelled material. This may not be a surface reaction since these sperm are readily damaged by handling.

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Studies of fibers of acto- and paramyosin from lamellibranch muscle. TERU HAYASHI, RAJA ROSENBLUTH AND HAYES C. LAMONT.

Johnson and Szent-Györgyi have proposed that the "catch mechanism" of lamellibranch muscle is due to crystallization of the paramyosin component of these muscles by a pH shift within the muscle, the crystallization effectively "freezing" the muscle at any state of contraction. The mechanism was based on studies of glycerinated muscle and the pH dependence of the crystallization of paramyosin from solution.

This hypothesis was tested by making fibers from mixtures of acto- and paramyosin in which the paramyosin content varied from ca. 20% (Prep. A: *Venus mercenaria* whole extract) to ca. 5% (Prep. B: *Spisula solidissima* whole extract) to ca. 1% (Prep. C: *Spisula solidissima* actomyosin) and testing the fibers for changes in ATP-induced contractability and mechanical stiffness with pH. It was found that ATP-induced contractability for all preparations at the constant ionic strength employed showed a maximum at pH 7.0. On the acid side (pH 6.5–6.8) Prep. A showed the greatest decrease of activity, Prep. B a smaller decrease of activity, and Prep. C the least decrease. Mechanical stiffness was measured only for Preps. B and C, by the application of quick stretches and noting the change in tension. It was found that Prep. B showed a much greater increase in stiffness below pH 7.0 than did Prep. C. It is concluded that, while more experiments are needed, the results of the present investigation tend to support the crystallization hypothesis.

A study of membrane elevation in various marine eggs. L. V. HEILBRUNN AND THOMAS F. BYERS.

We have studied membrane elevation in the eggs of the sea urchin Arbacia, the clam Spisula, and the worms Chaetopterus and Hydroides. In the Arbacia egg, membrane elevation is a normal process and is accompanied by a breakdown of cortical granules. In the other eggs studied, membrane elevation does not occur normally, but is initiated by the same types of agents as those which cause membrane elevation in the Arbacia egg and is probably due to the same type of mechanism.

Cortical granules are not essential for the process. In the Chaetopterus egg, the cortical granules can be centrifuged to one end of the egg and then ultraviolet radiation causes membrane elevation in that part of the egg devoid of cortical granules. Sometimes, especially at the end of the breeding season, strong centrifugal force will shift the cortical granules in the Arbacia egg, and then after insemination, membranes will become elevated from regions devoid of these granules.

In the Arbacia egg immediately after membrane elevation the elevated membranes can be pushed back to where they came from by hypertonic solutions, especially those of $CaCl_2$ and $MgCl_2$.

If eggs are first exposed to isotonic sodium citrate solutions for a few minutes, no membrane elevation is possible (Arbacia, Chaetopterus).

Membrane elevation can scarcely be due to the swelling or osmotic pressure of a protein colloid, for it can be induced by 100% ethyl alcohol (Arbacia, Chaetopterus, Spisula). In aging Arbacia eggs, it can also be induced by dilute solutions of mercuric chloride.

Indirect evidence indicates clearly that agents which cause membrane elevation release calcium from the egg cortex (Chaetopterus).

We conclude that membrane elevation is due to a release of calcium from the cortex and that the calcium which is set free then exerts osmotic pressure against the vitelline membrane and pushes it out from the egg surface.

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On the electron spin resonance of adenosine triphosphate. IRVIN ISENBERG AND ALBERT SZENT-GYÖRGYI.

Theoretical calculations by B. and A. Pullman show adenine to be a good electron donor since its uppermost filled energy level is high, indicating a low ionization potential. Work of this laboratory, currently in progress, indicates that the tri-phosphate group may be a good electron acceptor. Since adenosine triphosphate may fold so that the triphosphate group fits neatly over the adenine ring, the possibility exists that adenine may donate an electron to the triphosphate, forming a molecule in which charge is separated. Adenosine diphosphate does not permit such a folding.

Preliminary studies have been made on the possible spin resonance of ATP and ADP. Dry ATP powder *in vacuo* gave a low broad spin resonance signal. This signal was extremely complex and covered at least 600 gauss. This width is an order of magnitude above that which is ordinarily obtained for free radical signals. The half-width line broadening to be expected from two electrons separated 3.5 A is about 400 gauss. ADP did not present such a signal but gave only a low simple absorption line that was oxygen-sensitive and could have been produced by residual oxygen in the sample.

Aggregation and schooling in the marine snail, Nassarius obsoletus. CHARLES E. JENNER.

For the fourth consecutive summer a population of mud snails (*Nassarius obsoletus*) in Barnstable Harbor, Massachusetts, has been shown to undergo a change in distribution pattern . . . from dispersed, in which snails are present over extensive areas of the flat, to aggregated, with snails occurring in massive aggregations. This striking change occurs abruptly at the close of the egg laying season. In the aggregated state schooling behavior is observed in which snails move, often in large numbers, in the same direction. Motion pictures have been made showing this change in distribution pattern and also illustrating schooling behavior.

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Effect of temperature on electrical activity of nerve cord and cardiac ganglion of Limulus. PARASKEVI J. MAVRIDIS AND RICHARD C. SANBORN.

Electrical activity of the intermediate segments of the cardiac ganglion and of the region of the ventral nerve cord between the circumoesophageal ring and the first abdominal ganglion was recorded at various temperatures. At all temperatures, the activity of the ventral cord was less in posterior regions than in the region proximal to the ring.

As the temperature of the cardiac ganglion was lowered, the frequency of bursts of electrical activity decreased from 17/min. at 25° C. to 9/min. at 10° C. and 6/min. at 6° C. Simultaneously the duration of each burst increased from 0.2 milliseconds at 25° to 0.6 milliseconds at 10° C. As the ganglion is warmed, the burst frequency increases until at 33° C. it is 23/min.

The behavior of the nerve cord is strikingly different. As the temperature is lowered from 25° C. the magnitude of the potentials recorded by external electrodes increases markedly. Thus, a typical cord displayed spikes with a maximum amplitude of 75 μV. at 25°, and spikes with amplitudes of 200 μV. at 10° C. As a cord is rewarmed, both the magnitude of the potentials and the frequency of bursts increase. There is a "hysteresis" effect, in that the frequency and magnitude of the bursts are higher in a cord which has been cooled than in one which has been maintained at a given temperature. Similar, though lesser, hysteresis occurs in the cardiac ganglion.

At temperatures higher than 33–35° C. both the cardiac ganglion and the ventral nerve cord display no activity. (Aided by a grant from the Purdue Research Foundation.)

Studies on the fertilization inhibiting action of Arbacia dermal secretion. CHARLES B. METZ.

Following certain conditions of stress *Arbacia* release a yellow-green fluid. This dermal secretion strongly inhibits fertilization (Oshima, 1921; Pequegnat, 1948) and fertilizin agglutination of sperm. The latter action involves inactivation of fertilizin combining sites (Metz, 1959). In view of this dual action it seemed of interest to investigate the mechanism of fertilization inhibition.

Dermal secretion was prepared by immersing *Arbacia* in tap water, rinsing them in sea water and collecting the resulting secretion. The pH of this dermal secretion was adjusted to 8.0. Inhibition of fertilization does not result from irreversible action on sperm for sperm washed from dermal secretion have undiminished fertilizing capacity. However, washing treated eggs seldom completely restores fertilizability although partial recovery with delayed cleavage is sometimes obtained.

Evidently the inhibiting action is upon the egg and involves the initial stages of fertilization. To localize the site of action of the dermal secretion, the egg surface was subjected to mild dissection and then tested for sensitivity to the dermal secretion. The mildest agent used was acid sea water. This removes the egg jelly. The fertilizability of such jellyless eggs, like control eggs, is reduced by dermal secretion. Evidently, then, the dermal secretion does not inhibit fertilization by action on the egg jelly. A further dissection of the egg surface is achieved by proteolytic enzyme treatment. Eggs treated with crystalline protease or trypsin followed by trypsin inhibitor (ovomucoid) and subsequently exposed to dermal secretion fertilize as readily as controls. They are insensitive to the fertilization inhibitor. Presumably the protease removes a site or substance with which the dermal secretion combines to block fertilization. This substance is evidently essential in normal fertilization. It probably is not fertilizin, for an excess of fertilizin fails to reduce the fertilization inhibiting action of dermal secretion.

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A light and electron microscope study of Limulus gill cartilage. PHILIP PERSON, DELBERT PHILPOTT AND ETHEL SUBEN.

In the light microscope, gill cartilage from young Limulus (1–2″ body diameter) is composed of small, closely packed cells (12–29 μ diameter) with deeply basophilic nuclei. A thin eosinophilic matrix surrounds all cells. With increase in body size, cell diameters and amount of matrix increase. In an 8″ animal cells of 250 μ diameter may be found. Young and old cells contain much water-soluble, alcohol-insoluble material. Cell contents and matrix are PAS-positive. In the very large cells referred to above, cytoplasm becomes filled with granules and small vesicles. Younger cells contain large vacuoles. With aqueous fixatives cells drop out of sections in large numbers. Alcoholic fixatives improve cell retention but distort the matrix. Electron microscope study of young cartilage reveals typical mitochondria, golgi apparatus, and endoplasmic reticulum. As matrix is formed, it is first a thin seam of amorphous material which separates adjoining cells. On either side of this amorphous material, chains of rod-like micelles are formed in a lamellar, appositional fashion. These rods seem to take origin as elements of the endoplasmic reticulum and other cytoplasmic components, which, as they approach the periphery of the cell, give the appearance of being incorporated into the matrix. The lamellae form concentric layers. There are also fibrous elements apparently lacking any periodicity in the matrix.

The effect of various metabolites on x-irradiated Tetrahymena pyriformis. JAY S. ROTH AND EILEEN FIORENTINO.

Tetrahymena pyriformis W grown in 2% proteose peptone, 0.2% yeast extract solution for three days were collected and washed three times with distilled water by gentle centrifuga-

tion. Aliquots of the cells were placed in small plastic boxes and irradiated with 300,000 r. During irradiation the cells were cooled with ice. Controls were treated similarly. Three types of experiments were run: I, the cells were irradiated in the presence of metabolite; II, cells were irradiated and metabolite added immediately afterward (1–3 minutes), and III, cells were irradiated and metabolite added 20–30 minutes later. Aliquots of control and irradiated cells were transferred to Warburg flasks containing buffer, and O_2 consumption measured at 10, 60 and 120 minutes after a suitable equilibration period. The metabolites used were sodium acetate, butyrate and pyruvate adjusted to pH 7.4; final concentration, 0.035 *M*. The stimulation of O_2 uptake of unirradiated cells by these metabolites was 99, 123 and 22%, respectively, for acetate, butyrate and pyruvate compared to controls without substrate. For I above, cells irradiated in water showed a depression of O_2 consumption of 13, 30 and 38% at 10, 60 and 120 minutes, respectively, after irradiation, while cells irradiated in the presence of pyruvate showed changes of +80, +17 and −7% at 10, 60 and 120 minutes compared to unirradiated controls with pyruvate. Acetate was less effective in preserving respiration while butyrate had little action. For II above, pyruvate showed +42, −6 and −27% changes in respiration compared to −18, −30 and −42% for the water control. Acetate was effective, but less so than pyruvate, and butyrate had a slight protective action. In III, the protective action of pyruvate was considerably less than in II, although in some experiments it was almost the same. Cell appearance after irradiation correlated well with the protective action of the metabolites. (Supported by a grant from the U. S. Atomic Energy Commission.)

Cells of Limulus polyphemus in tissue culture. RICHARD C. SANBORN, JUDITH A. HASKELL AND FRANK M. FISHER, JR.

Cells which migrate from ovary, hepatopancreas, nerve, leg, and cardiac muscle have been cultured in hanging drops using a medium composed of inorganic salts, sugars, organic acids, and 5 to 10% sterile Limulus serum. Within a few hours after preparation, two types of cells appear at the margins of the explant of all of these tissues: (1) free, wandering, rounded cells in the body of the medium, and (2) spindle-shaped cells attached to the liquid-glass or liquid-air interfaces of the drop. Both of these cell types remain viable in culture for at least 30 days, but after 10 days increasing numbers develop granules and vacuoles. Free cells can be maintained in a healthy state for at least six weeks by the weekly replacement of the original explant with a fresh tissue mass.

These cells do not appear to be hemocytes (amoebocytes) which have also been maintained on clotted blood for as long as three months. When we use clotted blood as an explant under our cultural conditions, no free cells migrate into the medium, and the amoebocytes in the clot are smaller and more granular than the free cells which we have described. In cultures to which blood has been added in proportions greater than 10%, the blood contracts into a pseudo-tissue without cellular migration. Medium without blood has consistently stimulated earlier migration of cells from the explants.

Explants of peripheral nerve and muscle give rise to a preponderance of spindle-shaped cells while other tissues yield a higher proportion of round cells. Within a few days after preparation, acellular fibers appear at the margins of explants of hepatopancreas and ovary. As these grow, migrating cells appear to become entangled in this network. (Aided by grants from the National Science Foundation and the Purdue Research Foundation.)

The effect of urethan on the mitotic apparatus of the Chaetopterus egg. HERBERT SCHUEL.

Cleavage of the eggs of the marine annelid, *Chaetopterus pergamentaceus,* is inhibited when they are placed in a 1% solution of urethan (ethyl carbamate) in sea water 5 minutes after fertilization. Relative cytoplasmic viscosity determinations made with the hand centrifuge show that the "mitotic gelation" does not take place.

Eggs put into 1% urethan at all stages of the first cleavage cycle are prevented from dividing. If the eggs are allowed to reach the stage where about half of them show cleavage furrows before they are exposed to urethan, the furrowing process appears to continue. Two minutes later all the eggs have two quite distinct blastomeres and a polar lobe. How-

ever, the furrows soon regress and the cells assume a spherical shape again, so that 15 minutes later only a small proportion of the eggs are seen to have completed furrows. These few eggs probably had already completed cytokinesis when they were exposed to urethan.

Cytological observations were made on preparations fixed in Bouin's fluid, sectioned and stained with Heidenhein's hematoxylin. Eggs put into 1% urethan 5 minutes after fertilization show no signs of the mitotic apparatus at a later time when in the sea water controls it is completely formed. If eggs are exposed to urethan at a time after the mitotic apparatus had already formed and are fixed 5 minutes later, no trace of the asters, centrioles or spindles can be seen.

This work was supported in part by a grant from the National Science Foundation to Dr. L. V. Heilbrunn.

Melanophore dispersing action of ataraxic drugs. GEORGE T. SCOTT.

During the winter, in association with Miss Martha Robinson, the observation was made that meprobamate (Equanil, Miltown) would cause dispersal of pigment in frog melanophores, an effect antagonized by adrenin. The drug was completely refractory in the hypophysectomized animal, in the isolated frog skin test and when placed in Ringer perfusate.

A comparative study using six drugs was undertaken on *Rana pipiens*, the sand-dab *Lophosetta maculata* and the skate *Raja erinaceae*. The M.E.D. on the frog for reserpine, chloropromazine (Thorazine), methoxypromazine (Tentone), and meprobamate was 1, 4, 6, 30 mgs. per 100 gms., respectively; azacyclonal (Frenquel) and oxanamide (Quiactin) were not effective. M.E.D. on *Lophosetta* for reserpine, chloropromazine and meprobamate was 0.7, 2, and 30 mgs.; azacyclonal, and oxanamide were likewise non-effective. M.E.D. on *Raja* for reserpine was 0.4 mg. Time required to cause dispersion of melanophore pigment was from 1 to 6 hours. Transfusion experiments revealed that 1 ml. of blood from a drug-darkened *Lophosetta* will cause darkening in a pale recipient which lasts for several hours. Two-tenths cc. of blood from a reserpine-darkened skate will cause expansion of frog melanophores. Melanophores of *Lophosetta* are unresponsive to injections of Pitressin or Pituitrin.

The experiments suggest strongly that in the frog and skate certain ataraxics cause secretion of intermedin. Since the melanophores of *Lophosetta* are apparently similar to those of the flounder *Pseudopleuronectes americanus* (Osborn, 1939) in being unresponsive to pituitary extracts, the source and nature of the active substance is uncertain. A neuro-humor from the dispersing nerve fibers is suspected although a melanocyte-stimulating hormone may nevertheless be involved. Further investigation is planned.

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Progenesis in digenetic trematodes and its possible significance in the development of present life-histories. HORACE W. STUNKARD.

Progenesis is the precocious development of sexual maturity in juvenile animals. The metacercariae of digenetic trematodes sometimes become mature in their intermediate hosts, usually crustaceans, aquatic insects or tadpoles. Both asexual generations and gravid metacercariae of a species of *Asymphylodora* occur in the fresh-water snail, *Amnicola limosa*. Eggs from these progenetic metacercariae were fed to laboratory-raised snails. Sporocysts and rediae were recovered from these snails, proving that a vertebrate host is not essential for completion of the life-cycle. There is a question whether progenesis is to be explained as an atavistic reversion or merely an abridgment of usual developmental cycles.

Metagenesis was first recognized by Steenstrup in coelenterates and parasitic flatworms but actually, alternation of generations was initiated and perfected in plants. The introduction of asexual reproduction was essentially similar in plants, sporozoans and digenetic trematodes. In all, it evolved in a parasitic generation. There is a close association between endoparasitism, asexual reproduction and metagenesis. The Digenea have a common ancestry with the Turbellaria. In certain rhabdocoels, asexual reproduction regularly alternates with sexual reproduction. Others, probably related to the ancestors of the Digenea, are parasitic in mollusks and echinoderms. If the ciliated larvae of ancient turbellarians, on invasion of a mollusk, were to form cyst-like structures in which the germinal cells separated and developed

independently, a very efficient method of reproduction, polyembryony, would have arisen. This method is employed by the Digenea and progenesis may have completed the primitive life-history. With the advent of vertebrates, the former definitive hosts were eaten and the worms acquired new definitive hosts to which sexual maturity was deferred. As a result, the former definitive hosts were gradually reduced to intermediate, paratenic or transfer hosts.

On the significance of "voltage clamp" data in the "membrane oscillator." TORSTEN TEORELL.

The "membrane oscillator" is a model device aiming at a comprehensive understanding of the electrical behavior of excitable tissues. In its simplest form this model consists of: Compartment (1) containing N/10 NaCl (small volume)/ a negatively charged, porous membrane; compartment (2) containing N/100 NaCl. In the discussion which follows, a modification of this model was used which incorporated "leaky" pores as well. A steady D.C. current is applied with the cathode in (1) and the anode in (2). If the current density is increased in graded steps, the transmembrane potential undergoes oscillatory excursions, first damped, then above a certain current density ("threshold") undamped. The configurations of these oscillations are similar to those of the nerve action potentials. Arrangements in which the current is maintained constant are referred to as "current-clamp" experiments. Conversely, "voltage clamp" refers to an experiment in which the transmembrane potential is maintained constant and the current required to accomplish this is recorded. As the level of the maintained potential is varied in the model, the current undergoes a number of characteristic changes. In practically every respect these changes are similar to those observed by Cole, Hodgkin, Huxley, Katz, Tasaki and others on voltage clamp experiments on nerve tissue. (A representation of voltage clamp experiments as voltage current graphs also show a marked similarity with those obtained on physiological objects.) In all cases a short initial and terminal burst of current is observed, of opposite directions. The intermediate portion may show a "dip" below, or form a curve above the "resting level," or both. The current flow in the membrane oscillator reflects the changes in the internal membrane resistance. These changes are the resultants of the concurrent driving forces arising from electrochemical and hydrostatic pressure gradients. The initial and terminal current spikes, referred to as "capacity spikes" by many electrophysiologists, as well as the intermediate current curve are here due to resistance changes. In this respect, voltage clamp phenomena are simply interpreted by a straightforward application of Ohm's law. "Capacitive spikes" in the model are an expression of the time course of a resistance change and are accordingly only "apparent" (Teorell) or "anomalous capacities" (Cole). The characteristic "dip" often referred to as a "sodium inward current" has in the membrane oscillator no specific significance, it is merely one of many transitory states. The results suggest that alternative explanations of voltage clamp results are possible and that it may be advisable to search also for other hereto often neglected factors in the nerve mechanism such as water movements producing hydrostatic pressure changes across the excitable structures.

Nucleotide interactions associated with muscle actin transformation. KENNETH K. TSUBOI AND TERU HAYASHI.

Reactions in which the bound nucleotide of the muscle protein, actin, participate were examined using labelled ATP with the isotope P^{32} present in the two terminal phosphate groups. Polymerization (induced by salt) and further reversible depolymerization (induced by ATP in solution of low ionic strength) of actin solutions prepared from acetone dried muscle powder are associated with concomitant changes in the phosphorylated state of the bound nucleotide.

Polymerized actin (F-actin) and depolymerized actin (G-actin) contain bound ADP and ATP, respectively. Consecutive transformation of actin solutions from F- to G- and back to F- was found to occur without alteration in the amount of associated bound nucleotide. The mechanisms involved in the associated nucleotide changes were studied with the aid of labelled ATP. Examination of bound nucleotide on G-actin, following depolymerization of F-actin in the presence of externally added labelled ATP (APP*P*) and quantitative removal

of excess, free, non-bound nucleotides on Dowex-1, revealed the presence of an ATP of identical labelling (equivalent distribution of isotope and S.A. of P groups) as that of the externally added ATP. Reversible depolymerization of F-actin in these experiments occurs, therefore, with a concomitant exchange of bound ADP with an ATP from the medium. Repolymerization of G-actin containing the labelled ATP (APP*P*) and isolation of the resultant F-actin by centrifugation, yields a product containing just one-half of the original isotope. The polymerization reaction is therefore accompanied by a direct dephosphorylation of the bound ATP and a release of the terminal P group into the medium.

Quantitative techniques for the removal of free nucleotides from actin solutions without removal of bound nucleotide were developed as a part of these studies. In spite of the inability of Dowex-1 to remove measurable amounts of bound nucleotide from G-actin solutions over a one-hour period, a complete exchange of bound ATP with externally added labelled ATP was found to occur within this period. This apparent paradox is being further examined.

A convenient and simple method for preparing nucleotide labelled actin preparations (either G- or F-) for further studies has also been incidentally provided from these studies.

Polyploidy in Mormoniella. P. W. WHITING.

Normally females are diploid, males haploid. Three mutations to male diploidy have been found. No reduction occurs in spermatogenesis, whether haploid or diploid, so that all sperm from diploid males are diploid. These males are as highly fertile as normal haploid. Their numerous daughters, which are triploid, lay many eggs most of which fail to hatch, presumably aneuploid. From the few (presumably euploid) eggs of unmated triploid females there develop haploid and diploid males. Triploid females mated to haploid males produce diploid and triploid females from their fertilized euploid eggs. The polyploid stock is maintained by alternating generations. For the "odd" generations diploid males are crossed to normal diploid females; for the "even" the triploid daughters are isolated and bred unmated. Triploid females mated to diploid males would be expected to produce from their fertilized haploid and diploid eggs, triploid and tetraploid daughters, respectively. Preliminary cytological study indicates this to be the case. The problem of genetic markers for separating these two classes of females has not yet been satisfactorily solved. Mutant eye colors are used as markers to separate diploid from haploid males. By using diploid sires whose sperm will have no effect to change eye color, separation of triploid daughters from tetraploid has been made, a few of the latter having been obtained. Markers used for separating the sires from their haploid brothers are not as yet satisfactory. It is proposed to remedy this condition by use of a second genetics locus. The problem of sex determination is still unsolved for Mormoniella. Females develop from fertilized eggs, males from unfertilized, seemingly without regard to ploidy.

Oxygen transport—a new function proposed for myoglobin. JONATHAN B. WITTENBERG.

A study of the secretion of oxygen into the swimbladder of fish, presented at this forum last year, gave strong evidence for the idea that the active transport of oxygen is mediated by an intracellular hemoglobin present in the cells of the gas gland. The present work explores the possibility that the penetration of oxygen into cells from regions of higher to regions of lower oxygen concentration might also be mediated by intracellular hemoglobins, *e.g.* myoglobin.

Lankester, in 1896, had noted the presence of intracellular hemoglobins in many tissues of marine invertebrates. Dissection of the animals available at Woods Hole revealed that the hemoglobin-containing tissues were frequently thick or bulky structures. Diffusion of oxygen into these structures would appear inadequate to supply their respiratory needs, suggesting that tissue hemoglobins serve to transport oxygen into the depths of these tissues.

Hemoglobin was chosen as a model compound. The penetration of oxygen through hemoglobin containing agar membranes was measured with the aid of the teflon-covered oxygen electrode. The penetration of oxygen through the same membranes in the absence of oxy-

hemoglobin was measured in the presence of carbon monoxide which converts all of the hemoglobin to the carboxy form.

The rate of penetration of oxygen through membranes containing oxyhemoglobin was in many cases about 1.6 times as great as through the same membrane containing carboxyhemoglobin.

On the basis of this finding a new function, oxygen transport, is proposed for myoglobin. The mechanism of this transport is not apparent and is the subject of continuing studies.

PAPERS READ BY TITLE

Changes in cytoplasmic structure following fertilization in Arbacia eggs. R. D. ALLEN AND L. BUNIM.

The echinochrome granules of sea urchin eggs respond to the presence of fertilized cortex (*i.e.*, passage of the cortical reaction) by migrating from various regions of the cytoplasm to a sub-cortical location within the first 10 minutes following fertilization (Allen and Rowe, 1958). A plausible working hypothesis to explain pigment migration would be contraction of fibrils connecting the pigment granules to the cortex after fertilization.

It appears not to be possible to decide on the basis of centrifugation data in the literature whether or not such fibrils could exist in the unfertilized egg, although it has been shown that these granules exhibit zig-zag translatory movements in the cytoplasm as if so moved (Parpart). At the height of the breeding season, centrifugal accelerations as low as $140 \times g$ can sediment nearly all pigment granules and larger oil droplets without visibly sedimenting the lighter yolk. In some lots of eggs after the peak of the breeding season, many pigment granules remain suspended even after acceleration of $1800 \times g$ has sedimented the yolk; application of $4000 \times g$ displaces the pigment to the centrifugal pole, showing that it is still the heaviest particle present.

By applying various accelerations at different times between insemination and the completion of pigment migration, it was found that some pigment granules become either trapped in or attached to a subcortical gel layer as little as 30 seconds following insemination at 19–21° C. An increasing number of pigment granules remain in the cortex when eggs are centrifuged at $500–1900 \times g$ during the first 10 minutes after insemination. It appears probable that at least some pigment granules can migrate to the cortical region in a centrifugal field of $500 \times g$, or a stress of about 3 dynes/cm^2. The evidence so far is compatible with our working hypothesis.

The effect of starfish toxin on amebocytes. F. B. BANG[1] AND A. B. CHAET.[2]

The occurrence of a heat-stable, dialyzable toxin in scalded starfish (*Asterias forbesi*) has been demonstrated previously. The present study concerns the effect of this toxin on coelomic fluid amebocytes of the starfish.

Through the aid of properly transmitted light, *in vivo* observations of amebocytes circulating through the dermal papillae were possible. Toxin injections (insufficient to induce autotomy) induced clumping of circulating amebocytes—a reaction reversed within 30 minutes.

Phase contrast studies showed that amebocytes, when removed from starfish and mixed on glass slides with equal volumes of concentrated toxin, round up and fail to send out the usual pseudopodial processes. Frequently, these cells not only lose their granules, but become glassy in appearance. In medium concentrations there seems to be no perceptible difference between the activity of the amebocytes in control and toxic fluids. However, if still lower concentrations of toxin were used (10–20%), the amebocytes became much flatter and thinner than usual, and more cellular clumping occurred than in the controls. This concentration is approximately the same as the final concentration of toxin present in the *in vivo* experiments

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[2] Supported in part by National Science Foundation Research Grant G-8718.

noted above in which agglutination occurred. Amebocytes of the sand dollar (Echinarachnius), as well as the horseshoe crab (Limulus) were similarly affected by the starfish toxin.

Amebocytes in coelomic fluid have been aseptically removed from starfish and kept alive in test tubes for at least two weeks (room temperature). Siliconization of glassware was not necessary, and the viability of cells was noted after two weeks by the pseudopodial processes sent out when placed on a glass slide, and by their ability to concentrate neutral red. Toxin (1–4%) had no demonstrable effect on the "cultured" amebocytes even after one week, but the cells were susceptible to 50% toxin. As in the *in vivo* experiments, these effects were reversible.

Experimental modification of the lunar rhythm of running activity of the fiddler crab, Uca pugnax. MIRIAM F. BENNETT AND FRANK A. BROWN, JR.

Groups of male fiddler crabs, *Uca pugnax*, collected from Chappaquoit were exposed to a light intensity of approximately 100 ft.c. from midnight through 6 A.M. and to one of less than 1 ft.c. from 6 A.M. until midnight for three consecutive 24-hour periods. Chromatophores of groups of 20 of these crabs were staged hourly from noon until they averaged 2.5 on the Hogben-Slome scale on the third day. These chromatophore readings were compared with those taken concurrently for control crabs which had been collected at the same times as the experimentals but were maintained in the laboratory at a constant light intensity of less than 1 ft.c. Comparisons indicated that the phases of the 24-hour cycle of color change for the light-treated animals had been shifted to times 5 to 6 hours (average for four different series—5.56 hours) earlier than comparable phases for the controls. Eight crabs of each group were then placed in chambers of activity recorders and their spontaneous running was recorded continuously for periods varying from 9 to 24 days under constant laboratory conditions. All groups showed activity cycles characterized by two maxima (12 to 13 hours apart) which moved to later times on succeeding solar days at the average lunar rate. Comparison of phases of the cycle of activity for the control and light-treated crabs indicated that phases of this lunar-day cycle of running had likewise been shifted to earlier times, but by 4 to 5 hours (average—4.4 hours). No tendency for the peaks of activity of the experimental crabs to move to the times of maximal activity for the controls was noted during the four different periods in which this experiment was performed.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Phasing of the rhythm of running activity of the fiddler crab. MIRIAM F. BENNETT AND FRANK A. BROWN, JR.

On July 28, 1959 and August 18, 1959, fiddler crabs, *Uca pugnax*, were collected from the beaches of Chappaquoit and Lagoon Pond. At Chappaquoit low tides occur ± 1 to 2 hours of lunar zenith and nadir, and at Lagoon Pond phases of the tidal cycle occur approximately four hours later than the comparable ones at Chappaquoit. The spontaneous activity of 8 crabs of each collection was recorded continuously under constant laboratory conditions for 15 (series 1) or 8 (series 2) days. It was seen that the phases of the lunar-day cycle of activity of the animals from Lagoon Pond occurred about five hours later than those for the Chappaquoit animals on day one, the first complete 24-hour period of observation immediately following collection. After this time, the peaks of maximal activity for the Lagoon Pond crabs gradually moved toward the times at which peaks of running for the Chappaquoit animals occurred, and by the seventh day of observation were synchronous with them. Maximal activity for crabs from Chappaquoit occurs under laboratory conditions near the times of lunar zenith and nadir, or within an hour or two of the times of low tides on their native beach. During the later days of observation (series 1), the phases of the lunar cycle for the crabs from the two different beaches remained in synchrony with each other, and continued to occur within one to two hours of the times of lunar zenith and nadir.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Actions of biogenic amines and derivatives on lobster neuromuscular transmission. F. BERGMANN, J. P. REUBEN AND H. GRUNDFEST.

Short-chain omega-amino acids and some derivatives activate the inhibitory synapses, while another derivative—carnitine—activates excitatory synapses. Other biogenic amines, *e.g.* epinephrine and norepinephrine, are inert even at 10^{-4} dilutions. Serotonin (5-HT) appears to enhance transmitter action, for, while the excitatory postsynaptic potentials (epsp's) are considerably increased, there is no significant change in resting potential or membrane conductance, such as might be caused by a synapse activator drug. Homologs produced by methylation of the terminal amino group, or the alpha-carbon, behave similarly. The phenolic hydroxyl may be largely responsible, since tryptamine and its monomethyl homolog, after first increasing the epsp then depress it and the ipsp. Other homologs of tryptamine, and gramine, have only depressant actions. In small part, however, the creatinine moiety present in all crystalline preparations of the 5-HT series may contribute to their action. However, creatinine itself is weakly active (10^{-3} w/v), while 5-HT is effective at 10^{-6} dilution. PEA (beta-phenethylamine) also depresses both epsp's, but tyramine, which has the phenolic hydroxyl, is inert. PEA and 5-HT are antagonists with respect to effects on epsp's. These actions are probably mediated by effects on transmitter activity, since the interplay of the drug effects is not accompanied by changes in electrical properties of the muscle fibers. However, it is not known whether the various drug effects described above are caused by enhancement or depression of release of the synaptic transmitter, or by some action on the transmitter substance itself.

Contribution of locomotion to the oxygen-consumption rhythm in Uca pugnax. WILLIAM J. BRETT, H. MARGUERITE WEBB AND FRANK A. BROWN, JR.

Crabs collected weekly at Chappoquoit were subjected in the laboratory to either normal light-dark changes or to reversed light-dark conditions. Both control animals and light-reversed animals were set up in respirometers and activity recorders maintained in constant low illumination. Averages for 12 to 14 crabs were used for all hourly values. The data were analyzed for possible solar and lunar cycles. The form of the average diurnal cycle for both locomotion and respiration for the 29-day period between July 21 to August 18 was just the reverse for the controls and the experimentals. In the controls the maximum for locomotion occurred at 2 A.M. and for respiration at 4 A.M. In the experimentals the maximum for locomotion occurred at 1 P.M. and for respiration at 3 P.M. Analysis of the daily records for oxygen-consumption and locomotion in controls and reversed crabs showed a lunar cycle with the two maxima generally 12 to 13 hours apart. The mean lunar cycle for the 29-day period revealed little if any difference between the controls and reversed animals in either oxygen-consumption or spontaneous activity. In oxygen-consumption one maximum occurred at one hour before lunar zenith in controls and experimentals, and the second maximum occurred at two hours before nadir in the controls and three hours before in the experimentals. For locomotion the maxima for controls and reversed animals occurred two hours before zenith and two hours before nadir. A coefficient of correlation of $+0.919 \pm .033$ ($N = 24$) between the control animals and $+.545 \pm .146$ ($N = 24$) between the reversed animals for the average diurnal cycle indicates that locomotion produces a considerable effect upon the form and magnitude of the oxygen-consumption cycle.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

A diurnal rhythm in response of the snail Ilyanassa to imposed magnetic fields. FRANK A. BROWN, JR., H. MARGUERITE WEBB, MIRIAM F. BENNETT AND FRANKLIN H. BARNWELL.

Ilyanassa were permitted to emerge from a narrow corridor into a symmetrically illuminated white field, under conditions of three kinds: without imposed magnetic field and with each of two kinds of imposed fields, one with lines of induction parallel to, and the other with

lines at right angles to the initial path of the snails in the corridor. For each of 483 series of 60 snail-runs, two groups of ten in each field, obtained during the period July 6 through August 6, 1959, (a) the mean path of the snails in each magnetic field was expressed as difference from the control, and (b) the standard deviation of each was similarly expressed as difference from the control standard deviation. There was a daily rhythm in each of these differences. In the amount of turning, the snails turned to the right of the controls in the early morning hours, and to the left of the controls the rest of the day. The forms of the daily cycles for the two magnet orientations were very closely similar. In standard deviations, for both magnet orientations there were, parallelly, three sharply delimited periods of less deviation than for controls (5 A.M., 12 noon, and 7 P.M.) and more deviation than control for the other fourteen hours. For both of these effects of imposed magnetic field there was indication of an ability of the organism to differentiate between the two orientations of the imposed magnetic field, as shown by systematically differing relative influences of the two magnetic conditions as functions of time of day.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Effects of imposed magnetic fields in modifying snail orientation. FRANK A. BROWN, JR., MIRIAM F. BENNETT AND WILLIAM J. BRETT.

An 18-cm. Alnico bar-magnet (equating at about 40 cm. the earth's field) was placed horizontally on a turn-table 14 cm. beneath an "orientation chamber" with the center of the magnet directly beneath the exit of a 3-cm. corridor from which snails were emerging. The amount of the deviation of the snails from a straight forward course was determined. During the period July 6 through August 6, 1959, 483 series of 60 snail paths were assayed between the hours of 5 A.M. and 9 P.M. Each series (60 snails) consisted of two groups of 10 snails without magnet present, two groups with magnet at right angles (south pole to right), and two groups with magnet parallel and south pole directed away from exit. The order in series was continually changed. For most of the period of study, the observer was uninformed as to the presence or absence, or orientation, of the magnet. Analysis of the results indicated decisively that the snails responded to the presence of the magnet. This was indicated in each of two ways, both with $p < .005$ using all data, and with a $p < .001$ when only data obtained between 7 A.M. and 6 P.M. were used. The first way was in the mean amount and direction of turning. The snails in the imposed magnetic fields turned more strongly to the left, on the average, than in the natural magnetic field. The second was in the dispersion of pathways expressed as standard deviations of the three groups of 20 in each series. Standard deviations averaged significantly greater in animals in imposed magnetic fields. Orientation of the experimental chamber in different compass directions did not alter the major features of turning which were characteristic of any given time.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Fluctuations in the orientation of the mud snail, Ilyanassa obsoleta, in constant conditions. FRANK A. BROWN, JR., WILLIAM J. BRETT AND H. MARGUERITE WEBB.

Snails collected at intervals of 4 to 5 days from mud-flats at Chappaquoit were studied in white-enamelled boxes designed to measure the right and left turning of snails as they emerged from 3-cm. long corridors directed toward the magnetic south. Each box was illuminated by a large, diffusing light source located symmetrically above and slightly ahead of the corridor exit. Illumination from above was about 300 lux and from ahead and right and left, about 150 lux. Samples, each consisting of 10 snail exits, were taken between 5 A.M. and 9 P.M. during the period July 6 through August 6, 1959. The average angular deviation of the path from the straight line, measured for each of the 10 snails as they crossed a semi-circular arc with a radius of 3 cm. centered at the corridor exit, was determined. During the month of study there was a significant mean daily rhythm in the amount of turning with minimum at 5 A.M. and major maximum at 9 P.M. There was a secondary minimum

at 6 P.M. There was also a highly significant daily rhythm of total dispersion of pathways with a minimum dispersion at 5 A.M., a gradual steady increase to 10 A.M., and then a steady decline to 9 P.M., the time of a second minimum in the daily period of observation. Finally, there was a gradual irregular increase in the mean daily amount of left-turning, during the 32-day period. The form of this fluctuation suggested a two-day lead correlation on mean daily barometric pressure. A coefficient of correlation of 0.4 ($P < .05$, $N = 30$) was found between these.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Neural involvement in the firefly pseudoflash. ALBERT D. CARLSON.

When a firefly is made hypoxic it develops an "hypoxic glow," and if it is then returned to air a bright "pseudoflash" usually occurs. According to the tracheal end-cell valve theory the pseudoflash is due to entry of oxygen through temporarily inactivated valves (*i.e.*, is independent of neural control). However, neural involvement in the pseudoflash is indicated by the following facts: (1) Under standard conditions a pseudoflash of high intensity can be induced in 5 seconds in a firefly which has been actively flashing spontaneously, but requires 30 seconds in a quiescent individual. (2) When pseudoflashes are induced repeatedly in a firefly the intensity of the successive pseudoflashes decreases progressively and the time required to reach maximum hypoxic glow intensity increases. However, if the specimen is stimulated electrically or mechanically while in air between bouts of hypoxia, the intensity of successive pseudoflashes and rate of rise of the hypoxic glow can be maintained. (3) Low frequency stimulation during early hypoxia increases the hypoxic glow intensity. (4) Pseudoflashes can be more rapidly induced in a decapitated animal by injection of 10^{-3} *M* serine than in an uninjected animal. (5) Transection of the nerve cord between the fifth and sixth abdominal ganglia greatly reduces the pseudoflash in the regions of the lantern innervated from the sixth, but leaves unaffected those regions innervated from the fifth.

Peripheral aspects of firefly excitation. JAMES F. CASE AND JOHN BUCK.

Even with electrodes directly in photogenic tissue, the response latency of the adult *Photuris* is 65 milliseconds at 25°. This long delay is especially curious in that the actual light emission, involving coordination of several hundred thousand photocytes, may occupy only 50 msec. The possibility of at least two steps in the activation of the lantern is suggested by the fact that an intense stimulus (for example, 5–10 msec., 60–150 volts) may induce a short-latency flash (15 msec.) in addition to the usual long-latency flash, the minimal stimulus for which is 1 msec., 3 volts. This "quick flash" shows the same sorts of facilitation and strength-duration relations as the normal (slow) flash, is of comparable intensity and form, and appears to involve the same tissue. However, the quick flash differs sharply from the slow flash in its temperature response, showing scarcely any change in latency, and it can "follow" a much higher frequency of stimulation. The quick flash might represent either direct stimulation of photocytes or the by-passing of some intermediate echelon in the activation sequence. However, the quick flash latency probably represents more than the actual chemical delay of photogeny, for the quick flash of the larva is essentially instantaneous. Since tracheal end-cells are absent in the larva it is tempting to guess that the first step in the activation of the adult lantern—the step by-passed by intense stimulation—is the nervous stimulation of end-cells which then coordinate the flashing of the photocytes in an as yet unknown fashion. With regard to differential responses at the effector level, we find that various agents such as hypoxia and eserine can dissociate the over-all flash into asynchronous sparkling of tracheal "cylinders" or even of individual photocytes.

Physiologic activity of nerve extracts. A. B. CHAET AND R. A. MCCONNAUGHY.

While working on unrelated problems, it was discovered that hot water extracts of starfish nerves (*Asterias forbesi*) induced the shedding of gametes when injected into small starfish (4–5 inch). After 15 radial nerves were isolated and washed, they were suspended in a test

tube containing 15 cc. of sea water. This supernatant was then heated (1½ minutes at 76° C.) and considered a control. The 15 nerves were re-suspended in sea water and heated (1½ minutes at 76° C.). This supernatant, the first extract, when injected into recipient starfish induced shedding in 36–39% of the animals. Each starfish received an extract obtained from approximately 5 radial nerves (0.15 cc./gm. starfish). It is significant to note that upon autopsy, only 10% of the gonads appeared to be "mature," whereas 52% were "very poor." This procedure did not extract all of the shedding substance, since a second extraction using the same nerves induced shedding in 38–41% of the animals. Various attempts at more efficient extractions have been unsuccessful.

The shedding reaction was not due to the presence of potassium since the shedding substance was heat-labile and non-dialyzable. Furthermore, direct potassium analysis has shown that some extracts contained no more potassium than controls and still they induced shedding.

Recipient starfish failed to respond to the shedding substance after the middle of July, due to the lack of gametes (almost all gonads were "very poor"). However, the possibility that the concentration of the shedding substance available in the nerve fluctuates seasonally is being tested.

When starfish were injected with the above extracts (either fractions 1 or 2), it was noted that 2.3–3.5 times more fluid was excreted by the experimental animals than the control. This diuretic action was not decreased when the extracts were autoclaved but was slightly lowered by dialysis.

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Relation of x-ray dosage to modifications in fertilization and early development of Arbacia punctulata. RALPH HOLT CHENEY AND CARL CASKEY SPEIDEL.

Systematic studies of effects of a graded series of x-ray irradiations were made, in each instance, upon gametes from a single pair of sea urchins. Dosages ranged from 0.5 to 600 kiloroentgens, principally 1, 2, 4, 7.5, 30 and 60 kr. Experimental material was exposed in "Position A" to cross-firing from Coolidge x-ray tubes yielding 4940 r per minute. Observations by each of us were made synchronously of mass cultures and, by special techniques, aliquots were examined momentarily with the compound microscope. Events were followed continuously for the first few hours, and later at frequent intervals during critical periods of differentiation of blastulae, gastrulae, and plutei. Irradiation effects were observable not only on the cells themselves but also on the surrounding jelly, as evidenced by adhesive clumping of the eggs accompanying higher dosages. Direct relationship existed between dosage and degree of retardation in streak formation, prophase amphiaster, first and subsequent cleavages, blastula, and later development.

Our method distinguished clearly between the higher dosages employed in each series. Appreciable deviation from normal development was detectable also after 0.5 kr. This indicated the sensitivity of *Arbacia* to irradiation. Extent of retardation and percentages of abnormal types resulting from changes initiated by x-ray irradiation were similar in many respects to those found by us in parallel experiments with 2537 Å ultraviolet irradiation. Such similarities of equivalent abnormalities gave an indication of comparable dosages of these two physical agents.

Illustrative ciné-photomicrographs were obtained. Pictures taken at slow speeds vividly revealed changes during early development; those at normal speed showed individual deviations in movement and structure during later development.

This investigation was supported by a research grant (PHS RG-4326 C2) to C. C. S. from the National Institutes of Health, Public Health Service.

A rapid method for determining respiratory quotients in small animals. C. LLOYD CLAFF, F. N. SUDAK, J. R. BLUMSTEIN AND F. J. TEYAN.

A method for the rapid determination of respiratory quotients of small animals is described. R.Q. measurements can be obtained every 20–25 minutes. The apparatus used consisted of a Pauling paramagnetic oxygen analyzer connected either in series or in parallel with a carbon dioxide analyzer (titrimetric). Using an open ended system, dry, CO_2-free air

was drawn past the animal, confined in a brass metabolism chamber, at the rate of 325 cc./min. A sample (100 cc./min.) of the effluent air was bypassed through the O_2 analyzer and directly analyzed for the partial pressure of oxygen. The volume of O_2 was calculated from the difference in the partial pressure of the O_2 in the affluent air (room air) and the partial pressure of the O_2 in the effluent air, times the air flow rate divided by the barometric pressure at the time of measurement. The latency of the system was 3–4 minutes. Partial pressure of O_2 was read every thirty seconds for a 5–6 minute period and the average partial pressure for this time period was used in the calculation of oxygen consumption.

The volume of carbon dioxide added to the effluent air by the animal was measured titrimetrically three minutes later, using a known concentration of barium hydroxide, and thymolpthalin as the indicator. The time required for a carbon dioxide analysis was 6–8 minutes, depending on the size of the animal. Alternate O_2 and CO_2 determinations were made in duplicate. The results of a typical experiment in the determination of R.Q's of a fasting and non-fasting 163-gram rat were: non-fasting R.Q, 0.970, 0.970; 48-hour fast 0.702, 0.726; non-fasting R.Q. 24 hours later, 0.964, 0.949.

Metabolism-body temperature relationships in cold acclimatized rats treated with 2,4 Dichlorophenoxyacetic acid ("2,4-D"). C. Lloyd Claff, F. N. Sudak, F. J. Teyan and J. R. Blumstein.

The effect of 2,4 Dichlorophenoxyacetic acid on chemical thermoregulation was studied using fasted rats acclimated to 2.0° C. (3-week acclimatized period). Unacclimatized fasted rats served as controls. The "basal" metabolic rate, as measured by carbon dioxide production, was greater (150%) in cold acclimatized animals. "2,4-D" had no effect on the increase in "basal" metabolic rate which occurs during cold acclimatization. Evidence is presented which indicates that "2,4-D" does not interfere with chemical thermoregulation when cold acclimatized rats are exposed to low ambient temperatures.

The irreversible effects of caffeine on striated muscle. D. M. Conway and T. Sakai.

Caffeine can activate striated muscle without significant change in membrane potential, or even after the resting potential has been completely abolished by isotonic K_2SO_4, as found by Axellson and Thesleff with the frog sartorius, and confirmed by us using this muscle, and also the retractor penis muscle of the turtle *Chrysemis picta*. It had earlier been observed in this laboratory that frog muscle completely depolarized by 120 mM/l. KCl goes into irreversible contracture, just as normal muscle does on quick freezing and subsequent thawing. We suspected, therefore, that the effect of caffeine may also be irreversible, which would seem to limit somewhat the significance of the above observations. Recovery in normal Ringer after treatment with isotonic K_2SO_4 alone is complete, as judged by the isometric tetanus tension. There is a significant loss of tension, however, after caffeine treatment. The loss of tension is proportional to the concentration of the caffeine and the duration of the treatment. There is 95% recovery of the resting membrane potential (measured with intracellular microelectrodes) after 20 minutes' treatment with normal Ringer following 95 mM K_2SO_4 contracture. However, after caffeine contracture there is great scatter of values after 30 minutes' recovery, ranging from 10% to 75% of the normal. The threshold concentration for contracture at room temperature is 0.5×10^{-3} w/v, but lowering the temperature decreases the threshold. No contracture is elicited by 0.25×10^{-3} w/v at room temperature unless the solution is cooled to 0–4° C. The membrane potential is only slightly lowered on caffeine treatment and lowering the temperature causes a further drop in potential, but the value still remains above the critical potential.

The effects of caffeine on single fibers of the frog sartorius were studied with the phase-contrast microscope. Isotonic K_2SO_4 was found to have no effect on the fibers, but a concentration of 10^{-4} w/v caffeine induces a succession of contraction waves, followed by spontaneous twitching. A third stage involves disintegration of fiber structure. These observations were substantiated by electron microscopy.

Procaine has no effect on the caffeine contracture, neither has curare.

The sensitivity of the non-propagating muscle to longitudinal current. D. M. Conway and T. Sakai.

Our laboratory provided evidence that non-propagating striated muscle can be triggered by longitudinal electric fields of about 4 V/cm. We suspected that the field requirement of these muscles was high because of their low sensitivity to current. We studied, therefore, the question of whether under appropriate experimental conditions this sensitivity can be increased. Using the retractor penis muscle of the turtle we found that when it was recovering in normal Ringer from K depolarization, while the propagation had not yet returned, an a.c. or d.c. stimulus of about 2 V/cm. will bring about a substantial shortening along the length of the muscle, as recorded by the "fluorescent marking" method of Csapo and Mashima. If the recovery of propagation is delayed by using low temperature, or by having the depolarizing solution Ca-free, the sensitivity of the non-propagating muscle to current is prolonged. The recovery solution need not be normal Ringer; it is sufficient to lower the [K], *e.g.*, from 16 to 12 mM/l. In this case stimulation with 2 V/cm. longitudinal a.c. between two platinum plate electrodes in a square chamber produces 75% of the maximum shortening along the length of the muscle, when it is still non-propagating, as tested by stimulating one end only.

It was found that if the muscle is treated with 10 mM K_2SO_4 solution it becomes non-propagating within 5 minutes, and a longitudinal field of 2 V/cm. a.c. results in about 60% of maximum shortening.

Also, if 10% methyl alcohol is put on the muscle for about 5–10 minutes, propagation is lost, but a stimulus of only 1.5 V/cm. produces almost 50% of the initial shortening. In this condition the membrane potential is reduced to 55 mV. Recovery of normal excitability and tension was complete in a few minutes on return to normal Ringer.

Latency period, contraction time and fiber diameter. D. M. Conway and T. Sakai.

A correlation has been suggested (Peachey, *Science*, 1959) between the rapidity with which the active state develops, the diameter of the myofibril, and the density of the endoplasmic reticulum surrounding it. We have examined the duration of mechanical latency and the time required for the development of maximum tension, and attempted to correlate them with fiber diameter and density of endoplasmic reticulum in a typically fast and a typically slow striated muscle, *i.e.*, the frog sartorius and the retractor penis of the turtle.

Both muscles were stimulated at 27° C. with a.c. 60 c/s, 1 V/cm. for 1 second. Two recording channels of an electroencephalograph were used, one leading from the transducer and recording the tension, the other registering the duration of the stimulus.

The latency period in the sartorius is about 15 msecs. and the maximum tension measured isometrically is reached at 100 msecs. The latency period for the retractor penis is about 60 msecs. and the maximum tension is reached only after the termination of a one-second stimulus.

Electron micrographs were made of the two muscles and it appears that the myofibrils in the retractor penis are of the same diameter or thinner and the endoplasmic reticulum is more dense than that of the sartorius.

The diameter of the uterine muscle cell is only about 10 times greater than that of the myofibrils of the frog sartorius, but the latency period of the uterus is more than 100 times longer than that of the sartorius. The time required for development of maximum tension is about 60 times longer.

A general theory of membrane elevation in marine eggs. Donald P. Costello.

During past years, there have been so many theories proposed to explain membrane elevation in echinoderm eggs that it is almost presumptuous to contribute more on this subject. However, as was long ago pointed out by Lillie, a comparative study of the cortical response to activation of eggs of animals of several phyla may lead to a more generalized view than is possible by studying only echinoderms. Thus far, we have studied various aspects of the cortical response to fertilization of Arbacia, Asterias, Echinarachnius, Dendraster, Nereis, Hydroides, Spisula, Diopatra, Chaetopterus, Urechis, etc., using such methods as alkaline

NaCl treatment and/or centrifuging. In Arbacia and related forms, the vitelline membrane elevates (Heilbrunn) while a colloidal material accumulates (Loeb) in the perivitelline space. The membrane subsequently hardens and thickens into the fertilization membrane. In Nereis, the jelly precursor granules of the "cortex" release material which accumulates as a jelly external to the vitelline membrane (Lillie, Just, etc.). Other forms vary differently.

Our general hypothesis assumes, as do many earlier workers, that the basic similarity involved is the release of a "colloidal" substance by the egg on activation. The differences between the various eggs (magnitude of, timing, or absence of membrane elevation) might be accounted for in terms of (1) differences of porosity (or permeability to the colloid) of the vitelline and/or other membranes, (2) their nature, thickness, elastic tension, toughness or strength, (3) the time of release in relation to time of fertilization, (4) amount of substance released initially or over a longer period, (5) whether release is from the entire egg cortex, from localized areas, or from local points varying with time, (6) the molecular or micellar size of the released colloid.

For example, no membrane elevation need occur in an egg with a very tough, non-elastic, non-porous membrane, whose tension might greatly exceed the order of magnitude of the colloidal osmotic forces involved.

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A fundamental difference in the vitelline membranes of the eggs of marine annelids. D. P. COSTELLO AND CATHERINE HENLEY.

Alkaline sodium chloride at a pH of 10.5, isosmotic with sea water, has been known for some years to be an effective agent for freeing certain eggs of their vitelline membranes. However, the eggs of the annelids studied thus far fall into two very distinct groups as regards vitelline membrane behavior in this medium. Fertilized eggs of Nereis and Diopatra, after treatment, show an elevation of the membrane. Then, as the solution is changed a few times (to eliminate the divalent ions of sea water), the widely distended membrane ruptures at one point, the egg rolls out, and the empty membrane, consisting of two separate layers which are usually clearly discernible, remains. These eggs do not form sticky aggregates during the membrane-removal process.

In the eggs of Hydroides (either fertilized or unfertilized), on the other hand, after two or three successive washings with alkaline NaCl, the vitelline membranes become sticky, the eggs clump together, the membranes elevate and then *dissolve* to allow the denuded eggs to drop from the aggregate.

Novikoff (1937), using an alkaline NaCl treatment for the eggs of Sabellaria, found that the eggs adhered to each other to form aggregates. Then, as the membranes dissolved, the aggregates broke up and released the eggs, with adherent jelly.

There is obviously a fundamental difference in the vitelline membranes if, in one case, they become sticky and dissolve in the alkaline NaCl, and, in the other, are distended by the colloidal material swelling under them but remain relatively intact with the same treatment. In both cases, some colloidal material swelling in the perivitelline space accounts for the membrane elevation occurring during treatment.

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Electrophoretic analysis of the distal retinal pigment dark-adapting and light-adapting hormones in the prawn Palaemonetes. MILTON FINGERMAN, WILLIAM C. MOBBERLY, JR. AND CHARLES W. PHILPOTT.

The object of this investigation was to determine the electrophoretic characteristics of the hormones controlling migration of the distal retinal pigment in *Palaemonetes*. Forty eyestalks were extracted in 0.2 ml. distilled water and centrifuged. The supernatant was applied with the aid of a hot-air blower to a one-half inch wide Whatman No. 1 filter paper strip. In some experiments 0.1 *M* sodium hydroxide-boric acid buffer of pH 9.0 was used and in other experiments 0.1 *M* sodium phosphate–0.05 *M* citric acid buffer of pH 5.2. The voltage used was 500 volts and the current 0.1–0.2 milliampere. Electrophoresis proceeded for two hours. The portion of the strip where the extract had been applied and the first

three inches on the anodal and cathodal sides of the origin were washed for 30 minutes in 0.3 ml. sea water. The material washed from the three parts of the strip was then assayed on one-eyed *Palaemonetes* kept in black enameled pans under an illumination of 33 ft. c. At pH 5.2 the dark-adapting and light-adapting hormones were electropositive. When both substances were present in the same extract, the light-adapting effect preceded the dark-adapting one. At pH 9.0 the light-adapting hormone was again electropositive. However, about one-third of the dark-adapting material had migrated toward the anode. Presumably, the charge on some of the molecules of dark-adapting hormone had been reversed. The isoelectric point of this substance is presumably near pH 9.0. In other experiments both the dark-adapting and light-adapting hormones were found to be inactivated by trypsin.

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Influence of long exposure to light and darkness upon the distal retinal pigment light-adapting hormone of the prawn Palaemonetes. MILTON FINGERMAN, CHARLES W. PHILPOTT, MURIEL I. SANDEEN AND WILLIAM C. MOBBERLY, JR.

One group of *Palaemonetes* was placed in darkness and another group in white enameled pans under an illumination of 33 ft. c. for 14 days. After this period of adaptation, the supraesophageal ganglia were dissected from specimens in both groups and extracted in sea water. The concentration was 10 organs per ml. The extracts were assayed for distal retinal pigment light-adapting hormone on one-eyed *Palaemonetes* in black enameled pans under an illumination of 33 ft. c. The supraesophageal ganglia of the *Palaemonetes* that had been in darkness contained a greater amount of light-adapting hormone than the supraesophageal ganglia of the specimens that had been in light. No significant difference, however, was found between the quantities of light-adapting hormone in the supraesophageal ganglia of freshly collected specimens and of the prawns that had been illuminated for 14 days. By use of a dosage-response curve it was found that the supraesophageal ganglia of the specimens that had been in darkness for two weeks contained at least four times as much light-adapting hormone as the supraesophageal ganglia of freshly collected specimens and of the specimens that had been illuminated for 14 days. This difference in quantity of light-adapting hormone is evidence for the fact that this substance in the supraesophageal ganglia is normally involved in regulation of distal retinal pigment migration.

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Hormonal regulation of the distal retinal pigment of the shrimp Crangon septemspinosus. MILTON FINGERMAN, MURIEL I. SANDEEN, WILLIAM C. MOBBERLY, JR. AND CHARLES W. PHILPOTT.

Extracts of eyestalks and supraesophageal ganglia, with the circumesophageal connectives attached, from *Crangon* were assayed on *Crangon* kept in black enameled pans under an illumination of 31 ft. c. Within this intensity range the distal retinal pigment was in a position intermediate between the fully light-adapted and dark-adapted ones. A light-adapting effect alone was apparent. However, when eyestalk extracts of *Crangon* were assayed on *Palaemonetes* a dark-adapting effect followed the usual light-adapting one. When eyestalks of *Palaemonetes* were assayed on *Palaemonetes* light-adapting and dark-adapting effects were found but when extracts of eyestalks of *Palaemonetes* were injected into *Crangon*, light-adaptation alone was observed. When eyestalks of *Crangon* were subjected to filter paper electrophoresis at pH 9.0 the light-adapting hormone was found to be electropositive and the dark-adapting substance electronegative. The quantities of both hormones recovered were small. No light-adapting hormone had migrated toward the anode. Therefore, the small amount of dark-adapting substance that had migrated toward the anode was not masked by a light-adapting one as must have been the case when freshly prepared eyestalk extracts of *Crangon* were injected into *Crangon*. The distal retinal pigment of specimens of *Crangon* kept in black containers under an illumination of 31 or 33 ft. c. showed a 24-hour cycle of migration. This pigment more closely approximated the fully light-adapted state at this light intensity range about 7–8 P.M. than at any other time of day and was least light-adapted about 4 A.M.

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A reliable collecting locality for Pelomyxa palustris. J. L. GRIFFIN AND R. D. ALLEN.

Pelomyxa palustris (Greef) (the type species for the genus *Pelomyxa*) is important not only taxonomically, but also as material for many kinds of cell research. This species of ameba may regularly be found in a shallow inlet, near Dillingham Avenue, of the large freshwater pond which lies between Lawrence High School and the center of the town of Falmouth, Mass. Mast and others have collected *P. palustris* at this locality since 1934. Many other ponds in the Falmouth region have yielded *A. proteus* and smaller species, but not *Pelomyxa.*

It would be desirable if more investigators were to study *P. palustris* and arrive at their own conclusions regarding the best taxonomic designation for the giant ameba, variously called *Chaos chaos, Chaos carolinensis, Amoeba carolinensis,* and *Pelomyxa carolinensis.* It seems to us superficial and misleading to classify the giant ameba as a *Pelomyxa* on the grounds that it is multinucleate. *P. palustris* and the giant ameba differ from one another so widely in form, manner of locomotion, nuclear morphology, cytoplasmic inclusions, preferred food, and enzyme content that it seems best to maintain the giant ameba in a separate genus, *Chaos,* until the application of modern methods has provided sufficient evidence with which to revise the currently rather confused taxonomy of the large, free-living amebae.

Glucose-6-phosphatase in sea urchin eggs. PAUL R. GROSS AND GILLES COUSINEAU.

Ca-Mg-free *Arbacia* eggs were homogenized in cold KCl containing 0.001 *M* EDTA. With this preparation as an enzyme source, it was possible to obtain zero-order liberation of inorganic phosphate from a reaction mixture containing histidine buffer at pH 6.5 and the sodium salt of glucose-6-phosphate. The homogenates were then fractionated by differential centrifugation, a procedure which yielded pigment, yolk, mitochondrial, and microsomal pellets. The final supernatant, remaining after sedimentation of the microsomes at 80,000 × g for 30 minutes, was retained as the "soluble phase." The pigment fraction was not used for enzyme assay, since the echinochrome interferes with the phosphate determination. The microsome fraction is a collection of small RNP particles and a few very small vesicles; it accounts for 6.8% of the cell's TCA-insoluble N. The mitochondrial fraction is composed primarily of mitochondria with a few contaminating microsomal particles; it accounts for 5% of the TCA-insoluble N. The yolk pellet, which has more than 30% of the TCA-insoluble N, contains yolk particles and a few mitochondria. The soluble phase contributes 9% of the cell's TCA-insoluble N. When these fractions were assayed for glucose-6-phosphatase activity, the following specific activities were measured (μg P/mg. N/25 min. incubation): whole homogenate, 18.6, soluble phase, 0, microsomes, 2.0, mitochondria, 16.0, and yolk, 30.7. The data indicate that the enzyme is localized to a considerable extent in the yolk particles themselves or in some contaminant of that fraction (other than mitochondria). They are in sharp contrast to the situation in mammalian liver, where the enzyme is almost exclusively microsomal. We relate this finding to the absence of a highly-developed membranous component of the endoplasmic reticulum in the *Arbacia* egg.

Ultrastructural changes in regenerating rat liver. PAUL R. GROSS, MURIEL FEIGELSON AND DELBERT E. PHILPOTT.

Cells of normal and 46-hour regenerating liver from male Wistar (Carworth Farms) rats appear to differ most clearly in the following ways: *Endoplasmic reticulum* (E.R.): Present in normal liver, confined to "patches" in the cytoplasm. Found frequently in parallel associations of cisternae. The entire complex (including RNP particles) is more electron-dense than the cytoplasmic matrix. In regenerating liver, parallel cisternal systems are rarely found; when found, the membranes undulate. E.R. membranes here are often closely associated with the outer membranes of mitochondria. The entire complex is *not* more electron-dense than the cytoplasmic matrix. *RNP particles:* In normal liver, these are most obviously associated with E.R. membranes, although some are free; many particles of similar size in

cytoplasmic matrix are less electron-dense. In regenerating liver, there is a marked increase in the total number of *free* RNP particles; these, profusely scattered in the cytoplasmic matrix, are quite as dense as those associated with the E.R. membranes. *Ground cytoplasm:* In normal liver, there are large "hyaline" areas of low density; in regenerating liver, the ground cytoplasm is as dense (because of the large number of RNP particles) as are the nucleoplasm and the mitochondrial matrices. *Free fat droplets:* These are almost never found in the cytoplasm of normal liver cells, but are quite commonly observed in the regenerating material. They are internally structureless and more dense (osmiophilic) than other inclusion bodies. *Nuclei:* These are spherical or ellipsoidal in normal liver, with one or two nucleoli of granular (150–350 Å), but otherwise irregular structure. In the regenerating liver, many nucleoli are encountered with puckered surfaces, surrounded by cytoplasmic matrix free of large particles. Dense, periodic structures are found within such nuclei, suggesting condensed or condensing chromosomes rather than nucleoli.

Functional complementarity of leucine mutants of Neurospora. S. R. Gross.

An analysis of sixty independent mutational events at the *leu*-3 locus of *Neurospora* indicates a complex intralocus functional heterogeneity. Although each mutation presumably leads to the loss of the same enzymatic function, the mutants fall into four categories with regard to the restoration of enzymatic activity in heterocaryons. Members of group A (31 mutants) do not demonstrate restoration of activity with members of groups B, C or D. Group B (13 mutants), on the other hand, in combination with members of group C (14 mutants) demonstrate a restored activity. Group D mutants (2) restore activity in combination with some members of groups B and C. It seems certain that specific reorganization of enzyme protein occurs in the cytoplasm of heterocaryons. A study of the spatial orientation of the four functional classes within the *leu*-3 locus as well as the specific alterations in protein structure is in progress.

Membrane resistance and rectification in muscle stimulated by rapid cooling. Rita Guttman and Leonard Zablow.

After rapid cooling from about 25° C. to 5° C., no significant change in membrane rectification or capacitance of *Mytilus* smooth muscle could be detected. However, preliminary experiments indicate a percentage increase in membrane resistance equal to or greater in magnitude than that which occurs when sea water is cooled through this temperature range. The measurements were made by displaying as a Lissajous figure the current through and the voltage across the muscle, one end of which was treated with sea water containing 20 times the usual amount of KCl. The current was of triangular wave-form, with a frequency of 5 cycles/second. The resistive component of the impedance was balanced out by a bridge circuit, leaving a distorted ellipse on the screen, whose shape depended on the capacitance and rectification, changes in which could thus be determined. Since it was not possible to complete these measurements until some seconds after the onset of contraction (rapid cooling is an effective stimulus for this muscle), this change may well be unconnected with activity. Our method did not permit observation of transient changes of resistance less than 0.5 second.

Since previous investigations by one of us (R. G.) indicated that stimulation of *Mytilus* smooth muscle by rapid cooling is accompanied by changes in demarcation potential, we hope to investigate the possibility of earlier and smaller transient changes in future work.

Del Castillo and Machne could detect no change in membrane conductance of single frog sartorius fibers (which cannot be stimulated by cooling) greater than that which occurs on cooling Ringer's solution. Elucidation of whether rapid cooling affects electrical parameters of *Mytilus* muscle, where cooling *is* an effective stimulus, may indicate whether the cooling stimulus affects the contractile mechanism directly or triggers it through membrane changes.

The effects of podophyllin on the fertilized eggs of Chaetopterus. Catherine Henley and D. P. Costello.

Fertilized Chaetopterus eggs were treated with various concentrations of podophyllin (the stock solutions of which were made up in distilled water), beginning 5 minutes after

insemination, for periods of 42 to 60 minutes. Cytological preparations were made of samples of all treated and control eggs.

With concentrations of 0.0055 to 0.1 mg./cc., a characteristic series of changes was observed. For the initial 90 seconds, the first maturation metaphase was unaffected. About two minutes after the beginning of treatment, however, there were many eggs in which the asters had completely disappeared, and by 5 minutes after the beginning of treatment, all asters and spindles had disappeared. About one minute later, the egg chromosomes were surrounded by a nuclear membrane; in some instances, the chromosomes remained oriented in a peripheral ring just inside the membrane, in other cases, they appeared to fuse with one another, so that fewer than the normal haploid number (9) were present. This condition persisted until approximately 35 minutes after the beginning of treatment, at which time the egg nuclear membrane became much less distinct and the basophilic bodies within the membrane (representing either separate or fused chromosomes) were seen to be passing *through* the membrane. This process continued until by about 50 minutes after the beginning of treatment, the basophilic bodies were lying scattered in the egg cytoplasm, and the nuclear membrane had entirely disappeared. No asters or spindles were observed. No further changes were noted after this period, insofar as the chromosomes were concerned, and there was no subsequent development of the eggs.

The sperm nucleus remained unaffected by the podophyllin for about 60 to 70 minutes, after which time the same process of basophilic body extrusion was observed as occurred in the treated eggs.

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Shape changes in fertilized Chaetopterus eggs treated with podophyllin. CATHERINE HENLEY.

Fertilized Chaetopterus eggs treated with podophyllin by the methods described in the abstract by Henley and Costello above showed a number of shape changes which were remarkable simulations of such changes as observed in normal, untreated eggs of this form, despite the fact that maturation and cleavage spindles and asters were completely inhibited.

With the lower concentrations of podophyllin (particularly with the 0.0055 mg./cc. dosage), a semblance of polar body formation was often observed. This was characterized by the segregation of the entire egg nucleus into a large exovate (considerably larger than the normal polar bodies) which usually remained attached by a stalk to the egg cytoplasm. Such "pseudo-polar body formation" usually occurred at the same time as the formation of a normal first polar body in the controls, *i.e.*, approximately 11 minutes after insemination (6 minutes after the beginning of treatment). The pseudo-polar body was often resorbed into the egg cytoplasm within a period of about 10 minutes after its initial appearance, and there was usually no repetition of the event at the time of second polar body formation in the controls. In some eggs, exovates occurred in which no chromatin was present.

Concurrent in time with the characteristic pear and polar lobe stages of the control eggs, there were very normal-appearing similar stages in the experimental group. The polar lobes were subsequently resorbed by the eggs and no further development occurred. Cytological examination of such treated eggs showed that in many cases, at least, the shape changes could be correlated in time with the release of the basophilic bodies from the egg nucleus, as described by us above.

With the higher concentrations of podophyllin, exaggerated membrane elevation was observed, but this was not as pronounced as that seen after other experimental treatments.

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Examination of some D.P.N.- and T.P.N.-dependent dehydrogenase activity before and after relatively high doses of x-irradiation of Spisula eggs. E. KIVY-ROSENBERG, J. CASCARANO AND G. MERSON.

Work on the quantitation of dehydrogenase activity, using INT as hydrogen acceptor, in developmental stages of Spisula was reported by us earlier. The study of substrate-dependent dehydrogenase activity has been continued and extended somewhat.

Since the quantities of eggs are so limited in this form, gametes from 4-6 clams were pooled for each set of experiments and a microchemical technique employed. Samples of a 3.5% homogenate of both uninseminated and inseminated eggs were incubated aerobically at 37°–38° C. for one hour in a medium containing one of a series of substrates including succinate as well as some with DPN as cofactor, namely alpha glycerophosphate, glucose, glutamate, malate, lactate, beta hydroxybutyrate, ethanol and some with TPN as cofactor, namely malate, isocitrate, glucose-6-phosphate. Each set had appropriate controls.

The formazan was extracted and the amounts of reduced tetrazolium measured photocolorimetrically. The activity was expressed as micrograms of formazan per milligram of protein. The malate-dependent dehydrogenase activity was greatest and alpha glycerophosphate next in order for the DPN-dependent dehydrogenases, whereas isocitrate-dependent dehydrogenase activity was highest in the TPN-dependent dehydrogenases with malate and glucose-6-phosphate less active but still high.

To determine the effect of relatively high doses of x-irradiation upon their substrate-dependent dehydrogenase activity, 60,000 r was delivered to uninseminated and inseminated eggs. A covered plastic container of 30 ml. eggs in water was irradiated for 12′ 9″. The experimental series was made up of five situations: uninseminated controls, uninseminated x-rayed, inseminated controls, inseminated x-rayed and uninseminated x-rayed then inseminated.

Results seem to indicate that on the whole, x-irradiation increases some of the substrate-dependent dehydrogenase activity in both uninseminated and inseminated eggs: *e.g.* in the cases of the 5 most active dehydrogenases. This is not absolute or completely uniform for all egg pools however. It is less uniform in the TPN than in the DPN series. In the future, individual Spisula will be checked with the most effective substrates.

Extraction of egg jelly precipitating agents from Arbacia sperm. KURT KOHLER* AND CHARLES B. METZ.

Agents from sperm which precipitate egg jellies have previously been prepared by heating (Frank, 1939), freeze-thawing (Tyler, 1939) and mild acid extraction (Tyler and O'Melveny, 1941) of sperm. Such precipitating action has been attributed to a specific sperm receptor substance, antifertilizin. Upon further examination it is now found that frozen-thawed extracts of whole *Arbacia* sperm, isolated heads and isolated tails all precipitate egg jellies. These extracts were obtained as clear supernatants after 20 minutes' centrifugation at 3000 × g. At higher centrifugation such extracts yielded a sediment. With the formation of such sediment, jelly-precipitating action disappeared from the supernatant. This result was obtained in five independent extracts, one centrifuged at 7000 × g for two hours, two at 26,000 × g for thirty minutes and two at 100,000 × g for one hour. In two experiments, one at 26,000 × g, the other at 100,000 × g, such loss of activity from the supernatant was not demonstrated possibly because of resuspension of some of the sediment. In one experiment the 100,000 × g sediment was resuspended in sea water and found to precipitate jellies. In two experiments heating to 100° C. destroyed the egg jelly precipitating action of frozen-thawed extracts. These observations indicate that the jelly precipitating agent in frozen-thawed extracts is associated with particulate matter that coagulates upon heating.

On the other hand extracts prepared by heating sperm and centrifuging at 3000 × g failed to yield sediments after centrifugation at 100,000 × g. In one experiment aliquot extracts of a single sperm suspension were prepared by both heating and freeze-thawing. The egg jelly precipitating action of the frozen-thawed extract was removed but the heat extract remained active after centrifugation at 100,000 × g. These observations suggest that the jelly-precipitating agent in the heat extract is not associated with particulate material.

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The calcium-deficient uterus. H. A. KURIYAMA AND A. I. CSAPO.

Tension and action potentials (with intracellular microelectrodes) were recorded simultaneously *in vitro*. The uterine muscle of rabbits and rats was studied. Strips from the same uteri were examined at the same time using the "sucrose gap" method (see J. Marshall in this

* Fulbright Fellow.

volume), to get an over-all picture of the relative changes in membrane potential and membrane activity. Spontaneous and electrically- or pharmacologically-induced mechanical activity were also recorded from an additional pair of strips of the same uterus, to determine electrical excitability, threshold, maximum tetanus, effective drug concentration, long range behavior, etc.

If a parturient uterus is given a low concentration of oxytocin (for example 5 μU/ml.) and the early phase of membrane and myoplasmic activity is recorded, one can observe the one-to-one ratio between these two events. One action potential triggers a twitch, a number of them with low frequency an incomplete tetanus, whereas a train of discharges of appropriate frequency gives rise to a fused tetanus. An oxytocin concentration of 50 μU/ml. maintains for hours regular and maximal electrical and mechanical activity. The membrane potential fluctuates between the trains of discharges and each train is usually preceded by depolarization.

If the uterus in a steady state of maximum activity is rinsed repeatedly with Ca-free Krebs the membrane potential drops below 30 mV, the spikes become smaller, fewer and less frequent, and in 15–30 minutes activity ceases altogether. In this state of Ca deficiency the oxytocin effect is reduced to an abortive contracture. A threshold concentration of Ca (for example 0.3 mM/l.) increases the membrane potential above 30 mV and small spikes appear. With this change pharmacological reactivity returns, but in this condition oxytocin increases the membrane potential slightly, giving rise to large action potentials forming regular trains. As before the membrane potential fluctuates between trains of discharges and each train triggers a myoplasmic response. Thus, depending on its initial value the membrane potential is adjusted by oxytocin and is kept in a critical range where spontaneous activity is maximal.

Progesterone *in vitro* (10 μg./ml.) reduces the amplitude of the action potentials. Tension is also reduced, whether spontaneous, electrically- or oxytocin-induced.

The "evolution" of membrane and myoplasmic activity of uterine muscle. H. A. KURIYAMA AND A. I. CSAPO.

Tension and action potentials (with intracellular microelectrodes) were recorded simultaneously *in vitro*. Spontaneous electrical and mechanical activity of the uterus was studied during sexual maturation, pregnancy and parturition. In parallel experiments, isometric tension alone was recorded, and electrical excitability, threshold, maximum tetanus, effective drug concentration, long range behavior, etc., were studied.

The uterus of immature rabbits (4 months old or younger) has a membrane potential less than 30 mV. The spontaneous membrane discharges are very small and irregular, triggering irregular or no mechanical activity. Oxytocin does not improve upon such activity but induces a slight contracture. Incomplete estrogen stimulation slightly increases the membrane potential, spike amplitude and regularity. But tension remains small, irregular and of high frequency, with no adequate resting periods between activity. Oxytocin only exaggerates this contracture tendency.

Prolonged estrogen treatment brings the membrane potential up to 45 mV. The amplitude of the action potential increases, forming more or less regular trains, which trigger more synchronous mechanical activity. Oxytocin improves upon the regularity of the trains of discharges, increases their frequency and duration, thereby increasing mechanical activity. During the third trimester of pregnancy the membrane potential increases to a peak value of about 60 mV at placental sites. During this period regular trains of action potentials are not observed at placental portions, and irregular trains are only seen after several hours of perfusion, when they trigger asynchronous activity. Until the thirtieth day of gestation the rabbit uterus does not respond to 500 mU/ml. oxytocin, whereas the parturient uterus is affected by 0.5 μU/ml.

Delivery occurs at a membrane potential of about 50 mV. Immediately after parturition the uterus exhibits very infrequent spontaneous activity and only about 12 hours post partum does activity reach a high frequency. At this time the membrane potential dropped below 50 mV. The climax in the evolution of membrane and myoplasmic activity seems to be reached, therefore, a few hours after delivery. This climax can be effectively precipitated earlier by oxytocin. The membrane potential is close to 40 mV, 32 hours after delivery. Spontaneous membrane and myoplasmic activity then become somewhat irregular and tension begins to decrease.

It appears that regular phasic mechanical activity is closely linked to regular trains of action potentials of appropriate amplitude and frequency. Such membrane activity is induced

by estrogen and if present may be improved upon by oxytocin. Progesterone, on the other hand, suspends such activity.

Glucose-6-phosphatase content of toadfish islet tissue. ARNOLD LAZAROW, PAULA MAKINEN AND S. J. COOPERSTEIN.

Histochemical studies by other investigators have suggested that the beta cells of the islets of Langerhans are rich in glucose-6-phosphatase. This is of particular interest in view of the known relationship of glucose to the insulin-release mechanism. The method for the direct chemical analysis of this enzyme has been adapted to a micro scale and applied to toadfish islet tissue. In this species the islet tissue is separated from the exocrine pancreas and concentrated into a segregated body known as the principal islet.

The liberation of inorganic phosphate from glucose-6-phosphate was measured at pH 6.5 following a 15-minute incubation period at 37° C. The reaction mixture contained 2-5 μl. of a 1 : 5 islet homogenate (or a 1 : 40 liver homogenate), 0.04 *M* glucose-6-phosphate, 0.007 *M* histidine, and 0.001 *M* Versene in a total volume of 30 μl. Comparative studies were also carried out in which glycerophosphate was substituted for glucose-6-phosphate. The results were expressed as mμg. of phosphorus liberated per μg. of tissue in 15 minutes and the figures represent the average of 5 separate experiments with each tissue and each substrate.

Liver liberated 5.7 mμg. of phosphorus when glucose-6-phosphate was the substrate, as compared to 0.51 mμg. when glycerophosphate was used. By contrast islet tissue liberated 0.36 and 0.35 mμg. from glucose-6-phosphate and glycerophosphate, respectively. These data suggest that, in contrast to liver, the liberation of inorganic phosphate from glucose-6-phosphate by toadfish islet tissue may be due to the action of a non-specific phosphatase rather than to the presence of a specific glucose-6-phosphatase in this tissue.

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Leucothrix mucor. RALPH A. LEWIN.

L. mucor Oersted is possibly a colorless member of the Rivulariaceae (Cyanophyceae), hitherto isolated in Europe and western North America. Thirty-six isolates were obtained from the surface of algae, especially *Callithamnion* sp., along shores in the region of Woods Hole and Penikese Island. Pure cultures were established by streaking on sea water + Tryptone (0.2%) + agar (1.0%) medium. Streptomycin could not be used in the purification, since 25 mg./l. proved inhibitory to *Leucothrix*. The strains isolated differ only in minor characters, *e.g.*, degree of fragmentation and gonidia production, but these differences persist in culture.

By varying the components of synthetic sea-water media, 3.0% NaCl was found optimum, though growth occurred in concentrations down to 0.4%. Both K and Ca are essential. The following basal medium was found satisfactory for aerobic growth when supplemented with suitable N and C sources: NaCl, 30.0 g./l.; KCl, 1.0 g./l.; $CaCl_2 \cdot 2\ H_2O$, 0.1 g./l.; $MgSO_4 \cdot 7\ H_2O$, 0.1 g./l.; K_2HPO_4, 0.1 g./l.; "Tris" buffer, 1.0 g./l.; Fe and other trace elements; pH 7.7. In 48 hours at 25° C. dense flocculent growth was obtained with Tryptone (5.0 g./l.) or Na glutamate (10.0 g./l.). In basal medium with optimum concentrations of Na lactate (10.0 g./l.) and NH_4Cl (3.0 g./l.), growth was less dense, but no further stimulation could be promoted by the addition of yeast extract and cobalamin. The following compounds, tested in auxanograms or at various concentrations in liquid media, could not be used as sources of C for growth: glycerol, mannitol; arabinose, fructose, fucose, galactose, glucose, maltose, mannose, rhamnose, sucrose; acetate, citrate, fumarate, glycollate, malate, succinate; asparagine.

The effect of indole acetic acid and serotonin on the calcium^{45} content of leaves of the fresh-water plants, Potamogeton crispus (L.) and Elodea canadensis (Michx.). BENJAMIN LOWENHAUPT.

Leaves of *Elodea canadensis* (Michx.) and *Potamogeton crispus* (L.) immersed in $Ca^{45}Cl_2$ solution acquire radioactivity which reaches an equilibrium or steady-state level in a few

hours. Previous work has demonstrated that leaves thus prepared are useful in studies of calcium transport and equilibrium. Indole acetic acid and serotonin, it was found, release some of the calcium^{45} radioactivity from such leaves into their ambient solution. Effects of light and pH are significant, but have not yet been worked out.

This study was initiated in collaboration with Dr. Richard Klein at the New York Botanical Garden.

The effects of calcium deficiency and oxytocin on the membrane activity of uterine muscle, as measured by the "Sucrose Gap" method. J. M. MARSHALL AND A. I. CSAPO.

It is possible to measure, with external electrodes, membrane resting and action potentials from a strip of muscle, assuming that the muscle acts as a core conductor and that the short-circuiting between the electrodes is negligible. The latter is accomplished by increasing the external resistance of the muscle between the electrodes by replacing the interstitial fluid with sucrose. This method, the "Sucrose Gap," permits a continuous recording of changes in membrane potential and in rates of action potential discharge for a period of several hours.

Strips of uterine muscle from rats and rabbits were used. Portions of the same uteri were studied with microelectrodes under identical conditions (Kuriyama and Csapo, this volume). There was a close correlation between the results of the two methods.

The membrane potential of the parturient uterus underwent rhythmical cycles of depolarization and repolarization, the frequency of which was related to the mechanical contractions. During depolarization action potentials usually appeared. If oxytocin was applied during the quiescent phase of the cycle (repolarization and no action potentials), the membrane would immediately depolarize and action potentials would arise. When the parturient uterus was repeatedly perfused with a solution containing no calcium, the membrane depolarized about 20 mv. and all action potentials disappeared. If a small amount of calcium was added to the perfusion fluid (0.25 m*M*/l) the membrane potential increased somewhat although no action potentials appeared. The addition of oxytocin under these conditions increased the membrane potential to a level where action potentials appeared.

When a strip of muscle from a parturient uterus was perfused, *in vitro*, with a solution containing progesterone (20 μg./ml.) the fluctuations of the membrane potential and rhythmical discharges of action potentials were abolished and the membrane potential increased slightly. Oxytocin under these conditions was ineffective.

Supravital dye studies on isolated cells of frog renal adenocarcinoma. G. M. MATEYKO.

Dissociated, living cells of the frog tumor (Lucké renal adenocarcinoma) were immersed in supravital dye solutions of the following dye series: acridines, quinone-imine dyes, mono- and dis-azo dyes. Characteristic neutral red-staining granules of these tumor cells appear to be basophilic (RNA) components. Of particular interest were the thiazine dyes, methylene blue, methylene violet, Bernthsen (deaminated methylene blue), thionine, and toluidine blue 0, which showed initially, pale blue nucleoli within colorless nuclei, lavender cytoplasm with many coarse, irregular purple granules, but unstained spherical (neutral lipid) granules. This metachromasia is evidence of a viable cell population, the selective dye accumulation being dependent upon an active cellular process. Deep orthochromatic staining of the nucleus and cytoplasm is characteristic of a moribund population. Acriflavine and acridine yellow, however, exhibit an intensive and persistent nuclear staining in living cells (even without ultraviolet illumination), whereas with most dyes, nuclear staining is indicative of cell death. Accordingly, selective dye pickup and subcellular segregation, in contrast to diffuse orthochromasia of necrotic cells, are characteristic of a living cell population. Viability may be established, therefore, by the thiazine dyes, as well as neutral red, and vital red.

Dye accumulations were strikingly similar immediately after cell separation, and after storage at 4° C. for 24 or 48 hours, even in tumors from different animals. In contrast to the above, the dye patterns of normal animals show little vital metachromasia, especially in the reduction or lack of coarse stained cytoplasmic granules.

This investigation was supported in part by a research grant (C-4410) from the National Cancer Institute, U.S.P.H.S.

The localization of some dehydrogenases in Echinarachnius parma. GERALD MERSON.

It had been demonstrated that malic and succinic dehydrogenase systems could be isolated during the development of Arbacia. Work in this form was done on homogenates with TTC (triphenyl tetrazolium chloride) as the hydrogen acceptor. The present work was carried out histochemically on *Echinarachnius parma*, using a newer and a more rapidly reacting tetrazolium salt, INT (2-p-iodophenyl-3-nitrophenyl-5-phenyl tetrazolium chloride) in an effort to localize the dehydrogenases above. Toward this end ten stages of development were utilized: unfertilized, and fertilized egg, 8-cell, early and late blastula and gastrula, and early and late pluteus stages. In each case about 60 specimens were incubated in one of five media: (A) sodium malate (.2 *M* made in Ca-free sea water) with DPN and INT; (B) sodium succinate (.05 *M* made in Ca-free sea water) and INT; (C) Ca-free sea water and INT; (D) Ca-free sea water; and (E) untreated sea water. The final dilution of INT was about 0.0125%. Ca-free sea water was used for improved solubility of INT. The embryos were incubated anaerobically for 1½ hours at water table temperature (19.5° C. ± 0.5° C.) and observations made within the following half hour.

One can demonstrate a clear-cut precipitate of reduced INT(formazan)1 starting at the early gastrula stage. This activity is seen in the succinic and malic dehydrogenases. It is localized in the two areas of developing primary mesenchyme. In the mid-late gastrulae, this activity is extended to include a semicircle of cells which joins the two isolated plaques. During the pluteus stage, this activity is seen in all the arms and along the margins between the oral arms. Embryos incubated in the Ca-free and INT control (C) demonstrate only a mild diffuse pink color. All media permitted organisms to remain viable although development did not progress in any except the untreated sea water. Thus it appears that the primary mesenchyme is a site of malic- and succinic-dependent dehydrogenase activity.

Early responses of alloxanized toadfish. P. F. NACE, C. YIP, I. SILKOVSKIS, M. MOULE AND J. MOULE.

Beginning May 22, at sea water temperature of 14° C., adult toadfish of both sexes were injected with alloxan, 16% aqueous, at 700 mg./kg., through the heart. At intervals 1 minute to 23 days after treatment, blood samples were taken and fish were sacrificed for histological study. About 500 fish were studied by modified Folin (Murrell-Nace) and Glucostat blood sugar procedures, with fixation, after sacrifice, of principal pancreatic islet, liver, kidney (with interrenal) and brain (with pituitary). A smaller number of treated and control fish were injected with tritiated thymidine or with cystine labelled with sulfur-35. Pooled samples of blood of treated and control fish were studied by electrophoresis.

Egg-bearing females did not exhibit the "hyperglycemia" characteristic of males in the breeding season or of both sexes later in summer, after spawning and after water temperatures had exceeded 18° C. Maintenance of spawned females in water cooled to 15° C. did not restore resistance to alloxan, nor did it restore the 30–35 mg.% intact blood sugar levels found July 13, at 20° C., toward the 65 mg.% values found at 14° C. in May. Daily treatment with estrogen for 7 days did not change blood sugar level, but thyroxine increased response to lower doses of alloxan. For intact fish, and for fish sampled more than 6 hours after treatment, Folin and Glucostat values were comparable. However, the Folin "hyperglycemia" seen 1 minute to 6 hours after treatment was not reflected in parallel Glucostat determinations. Electrophoresis revealed no substance in experimental blood implicable in this divergence. The absence of the response in egg-bearing females militates against the implication of alloxan, which is a Folin-detectable reducing substance.

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Phosphoprotein phosphatase in the ovary of R. pipiens. SYLVAN NASS.

Differential centrifugation of ovarian homogenates of *R. pipiens* indicates that phosphoprotein phosphatase is located primarily in the small yolk platelets. Most of the small en-

dogenous activity found in clean "large" yolk at the enzyme's optimum pH (5.0) can be removed by solubilizing in 0.4 *M* NaCl and re-precipitating by dialysis against 0.1 *M* NaCl or distilled water. Attempts to extract the enzyme from the small platelets have been only partially successful. After 0.4 *M* NaCl extraction the extracts and residues contain similar activities. This is a result of either or both of two factors: (a) the small yolk platelets are less readily soluble in high ionic strengths than are the larger platelets; (b) the enzyme is rapidly adsorbed on pigment granules. Uncontaminated pigment granules have no PPPase activity. Attempts to extract the enzyme from the total homogenate after removal of the enzymatically inactive soluble proteins were discontinued because the extracted proteins were incompletely re-precipitated upon dialysis. By contrast, clean yolk platelets, when extracted are completely re-precipitated. Approximately 10% of the total protein phosphorus of phosvitin, casein and yolk is hydrolyzable by the enzyme. Yolk heated to 80° C., β-glycerophosphate, ATP and RNA were not attacked by the enzyme. The addition of 1% RNA to a phosvitin-enzyme preparation prevented inorganic phosphate release, while with ATP there was a slight inhibition. Inhibition studies indicate that the enzyme has somewhat different properties from those published for mammalian PPPases. In agreement with reports on mouse liver PPPase, frog egg PPPase is most strongly inhibited by molybdate (50% at 10^{-4} *M*). Cu^{++}, Hg^{++} and Mn^{++} inhibited 100% at 10^{-2} *M*, 20% at 10^{-4} *M*. However, Ca^{++} and Mg^{++} had very little effect at 10^{-2} *M*. Thioglycollic acid, desoxycholate, ascorbic acid, hydrogen peroxide, oxalate, and phenanthroline were without effect although both activating and inhibiting effects were found with these compounds by other workers on PPPases of different origins. Additional studies are necessary before any interpretation of these results can be made.

Uptake of Zn^{65} and primary productivity in marine benthic algae. EUGENE P. ODUM AND ROGER W. BACHMANN.

The rate of uptake of zinc-65 from normal sea water and the primary production of ten species of macroscopic benthic algae were measured in light and dark bottles suspended in a running sea-water aquarium under constant light and temperature conditions. Uptake and production of one species (*Chaetomorpha Linum*) were also measured in bottles suspended at different depths in a marine pond in order to achieve a gradient of light intensities. Approximately 3 μc of Zn^{65} were added to each bottle containing 250 ml. of sea water and approximately 0.5 gm. dry weight of algae. Definite uptake occurred in the light, but no appreciable uptake occurred in the dark. The rate of uptake in the light was proportional to gross oxygen production which varied with the species and the light intensity. Species with high surface-to-volume ratios exhibited more rapid uptake of zinc and greater gross oxygen production per gram biomass than species with low surface-to-volume ratios. Uptake of Zn^{65} apparently depends on photosynthetic activity since no uptake occurred in the dark and the rate in the light was independent of the concentration of the isotope or carrier added to the water. Consequently, Zn^{65} has possibilities as a tool for the measurement of primary productivity.

Protoplasmic contraction and cell wall swelling in a marine alga. W. J. V. OSTERHOUT.

A marine alga, *Chaetomorpha Linum* Kützing, was used. These cells were smaller, younger and less resistant to external changes than the cells used in previous years, which may be responsible for the difference in the results.

(I) A string of living cells was transferred at room temperature (25° C.) from sea water to distilled water. A crescent-shaped swelling appeared on the surface of the cell wall and the string of cells moved by irregularly bending until one spot on each cell burst. The protoplasmic contents rapidly flowed out, forming a green mass outside at the point of exit, and the cell became practically devoid of protoplasm. When a solution of 0.05 *M* NaH_2PO_4 at pH 4.5 was used the same results were obtained except that the viscosity of the protoplasmic mass was so much greater that it formed a thick plug at the cell wall opening which prevented further exit of the protoplasm. The protoplasm remaining inside the cell markedly contracted after a few minutes, while the cell wall remained swollen. These results indicate

a difference in the mechanisms controlling the cell wall and the protoplasm. With the cells used in previous years there were no changes even two hours after the cells were transferred from sea water to distilled water. As above indicated this difference may be due to difference in the age and in the previous history of the cells.

(II) A string of living cells was heated in sea water at 50° C. for five minutes until the cells were injured and acid fuchsin penetrated the cells. The string of cells was transferred to sea water at room temperature (25° C.) and after thirty minutes it was placed in distilled water at 25° C. Swelling of the cell wall occurred but there was no bursting. The protoplasm contracted after about thirty minutes. In 0.05 *M* solution of phosphate buffer mixture at pH 6 the protoplasmic contraction occurred more rapidly than in distilled water but the difference in the rate was not so marked as in the case described in 1955 in this journal. This difference in the results may be due to the difference in the age and in the previous history of the cells. If 0.05 *M* solution of NaH_2PO_4 at pH 4.5 was used the protoplasmic contraction occurred more rapidly than with a solution of phosphate buffer mixture at pH 6.

Water relations in injury to Nitella cells. W. J. V. Osterhout.

Single cells of *Nitella flexilis* about seven centimeters long were used. A cell was divided into two parts, A and W, by a vaseline seal. The movement of liquid inside the cell was observed by following the movement of suspended particles. If water was placed at A and at W there were no changes. But when water at A was replaced by a toxic concentration (3 *M*) of ethyl alcohol solution a striking series of changes occurred. (1) A rush of liquid inside the cell from W to A occurred. This was due to the higher osmotic pressure of alcohol at A which extracted the water from the cell at A while the water entered the cell at W. Suspended particles and dissolved substances were carried with the liquid from W to A and accumulated at A where they could not come out of the cell. The rush from W to A soon stopped when the osmotic pressure inside the cell at A increased so that water ceased to go out of the cell rapidly at A. (2) Then the reverse rush from A to W began. This was due to the fact that alcohol now entered the cell faster at A than water came out of the cell at A. This rush soon stopped because the osmotic pressure of the cell at W increased when dissolved substances inside the cell accumulated at W since they could not come out of the cell along with the liquid at W. (3) Then a more violent rush began from W to A. This was due to increase in permeability of the cell to substances inside the cell at A caused by injury from alcohol at A. The contents poured out at A from the cell which collapsed and died.

These changes were seen when a stained cell was used. Normally, brilliant cresyl blue accumulated uniformly in the vacuole because in the form of ions the dye could not come out of the cell. (1) In the first rush the dye was seen moving from W to A where it collected. (2) In the second rush the dye began to move from A to W. (3) In the third rush the dye came out of the cell at A because the permeability to dye ions increased.

These results may be due to changes in concentration only in the immediate vicinity of one or both of the protoplasmic membranes (plasmalemma and tonoplast).

If a non-toxic solution of ethyl alcohol (0.5 *M*) was used another process occurred as reported in 1950 in the Journal of General Physiology.

A study of the soluble proteins of the adult squid lens. John Papaconstantinou.

The soluble proteins from the lens of the adult squid, *Loligo pealeii*, were fractionated by use of the anion exchanger, diethylaminoethyl cellulose (DEAE). The fibers from the peripheral and core regions of the lens were homogenized separately in .005 *M* phosphate buffer, pH 7 and the clear supernatant of this homogenate was applied to a DEAE column. The protein was eluted from the column by a stepwise addition of phosphate buffers in which there was a simultaneous increase in ionic strength and decrease in pH. Aliquots of 10 ml. were collected and analyzed for protein concentration. In all experiments 92–95% of the protein applied to the column was recovered. Analysis of the elution diagrams showed that 85% of the soluble protein from the peripheral lens fiber homogenate and 93% of the soluble protein from the core lens homogenate were eluted by the starting buffer, *i.e.*, .005 *M* phosphate buffer, pH 7. To determine whether this "break through" protein consisted of several protein

components, antibodies to the peripheral and core homogenates were prepared and each 10-ml. fraction in the "break through" region of the elution pattern was tested by the agar diffusion technique. It was found that only one very heavy band was formed when aliquots of the 10-ml. fractions were permitted to diffuse against antisera to the peripheral and to the core lens proteins. The combined DEAE fractionation and agar diffusion experiments indicate that there may be only one major soluble protein in the peripheral and core fibers of the squid lens. Further analysis by electrophoresis and ultracentrifugation will be done to support the data presented above.

A calculator to aid in the correction of astigmatism in electron microscopes. DELBERT E. PHILPOTT.

A circular calculator for correction of astigmatism has been made of plastic to aid in teaching and to speed up the correction process. A 5-inch clear plastic disk is placed over a 360° segmented circle and attached in such a way that it is free to rotate. The positions of the eight iron screws located in the objective pole piece are plotted around the periphery of the circle. An inked line is drawn through the center of the plastic disk with an arrow pointing out at each end, the tips terminating on the edge of the segmented circle. This line is labeled "astigmatism produced." To make this calculator, the angle between screw direction and astigmatism is now determined in the regular way by sufficiently moving one pair of screws to produce a large increase in astigmatism. When this angle has been determined, the "astigmatism produced" line is set in the direction of the astigmatism and the screw direction which produced this astigmatism is plotted on the surface on the plastic in the form of arrows pointing inward. Using this calculator to correct astigmatism, set the "astigmatism produced" line at 90° to the astigmatism which is present in the microscope. The screws which must be turned in for correction are now read directly below the "screw direction" arrows. This system helps to obviate such things as uneven screw tips and screw wobble which can cause the astigmatism to change direction inconsistently causing confusion in the compensation. In this laboratory the screws are used to fully compensate the electron microscope and the electrostatic compensator is used to correct any astigmatism produced by the objective aperture. Otherwise the two vectors must be corrected at one time. Hence, two systems of correction again simplify the operation of the electron microscope. The increased visualization is also a distinct aid in teaching. The above procedure has been applied to electrostatic compensation with equal results.

An electron microscopic study of the sperm of Limulus polyphemus. DELBERT E. PHILPOTT AND WILLIAM N. SHAW.

The sperm of *Limulus polyphemus* was fixed in osmic acid, embedded in methacrylate and sectioned for electron microscopy. The anterior end of the sperm head consists of an acrosome cap, and a "small" axial body (a larger one was described in oyster sperm). An axial core traverses the head centrally from the small axial body to the region of the centriole in the distal end of the head. Immediately adjacent to the region of the centriole the axial core, or its continuation, bends laterally to the outer surface of the head and appears to be continuous with the coiled structure which forms approximately six closely adhering spiral turns around the base of the head. This posterior spiral seems to originate from the axial core but the axial core is hollow and the spiral is uniformly osmiophilic and apparently solid. Fitting up against this spiral coil and covering the posterior end of the head are numerous small mitochondria.

Normally the sperm flagellum arises from a basal structure which is made up of two centrioles and the origins of the filaments comprising the tail. A centriole region has been observed in Limulus sperm, but two distinct centrioles have not been resolved.

The tail proper is composed of a pair of central filaments and an outer ring of nine pairs of filaments. The central filaments stop just before they reach the attachment to the head; the outer filaments continue up into the head. The result is a weak point in the attachment region and any change in osmotic pressure breaks the tail at this point.

The entire sperm head is covered by an outer membrane which continues posteriorly beyond the mitochondrial region of the midpiece for a distance of .8 μ and then invaginates for the same distance to the proximal region of the tail. The posterior extension thus formed contains a spiral or possibly metameric structure which probably serves as a stiffening agent for the region of the tail attachment. The discharged sperm were examined for acrosomal filaments and several sections showed this filament to extend from the small axial body directly through the acrosome cap and out into free space. Further work is being continued on this material.

Chemical excitation of presynaptic terminals at lobster neuromuscular junctions. J. P. Reuben, F. Bergmann and H. Grundfest.

Although picrotoxin selectively blocks inhibitory postsynaptic membrane of decapod muscles, it also sensitizes terminals of both excitatory and inhibitory axons to other drugs: *e.g.*, beta-phenethylamine, serotonin, tetraethyl- and tetrabutylamine, and veratrine. Spontaneously, or after a single brief stimulus, an axon fires repetitively at rates up to 50/sec., often for several seconds. The "antidromic" impulses thus produced propagate to stimulate other muscles supplied by the axon. The repetitive activity is shortened, or stopped by GABA. Then, addition of more picrotoxin restores it, the interaction apparently being due to a competitive antagonism of the effects of picrotoxin and GABA. Several varieties of data localize the site of these actions to the nerve terminals. Applied to the axon itself, the drugs prolong the spike, or block it, the combination picrotoxin-serotonin being about equally effective as the picrotoxin-phenethylamine combination. Nor is the muscle directly involved in the drug-induced repetitive activity of the axon. The individual axons fire at different rates and asynchronously, repetitive activity in one axon not affecting that of the other in the case of the stretcher innervation. When muscles are supplied with more than one excitatory axon the thresholds of the drug effects are different. The membrane resistance and resting potential of the muscle fibers are not changed by the drugs prior to the onset of the repetitive firing of the axons. The electrical excitability of the muscle fibers may be eliminated, as by selective depolarization by glutamate, without affecting the axonal discharges. Therefore it seems likely that the picrotoxin sensitizes the nerve terminals to the other drugs which then depolarize the terminals. The resulting "generator" potential would then tend to fire the axons. The reversal of the picrotoxin action by GABA forms a striking parallel to the antagonism of these two substances on lobster neuromuscular synaptic membrane or axodendritic synapses of the cat cortex. However, the nature of the action is different in each case.

Anomalous current-voltage relation induced by hyperpolarization of lobster muscle fibers. J. P. Reuben, R. Werman and H. Grundfest.

Intracellularly applied hyperpolarizing pulses cause a discontinuous change in the membrane potential. At a threshold of about 70 mv hyperpolarization, and while the current remains constant, a "response" begins with variable latency, rising slowly to about 200 mv hyperpolarization. Stronger currents shorten the latency. The response can be induced by adding a brief anodal pulse to a subthreshold current. It can be abolished by cathodal pulses in all-or-none fashion, indicating a range of forbidden potential between threshold and response peak. The response is variable in form and duration. Sometimes it persists, with or without small oscillations, throughout the applied current, or it may terminate earlier, the potential returning to approximately the threshold value. In the latter case, several more or less identical responses may occur. Measured by amplitudes of applied brief pulses, or of evoked epsp's, the membrane resistance at the response peak is 3 to 4 times higher than at the threshold. Summated ipsp's restore the membrane potential approximately to the Cl equilibrium potential. Probably, therefore, neither synaptic membrane nor Cl-permeability are involved in the response. Addition of Ba, or of high Ca, which augments membrane resistance by blocking K-permeability (Werman and Grundfest, below; Reuben, unpublished), abolishes the response, as does $2 \times K$; the divalent ions by raising the membrane resistance as high as or higher than that at the response peak, while K raises the threshold. Thus, the response is probably caused by an

abrupt, temporary decrease of K-permeability. At threshold currents the resistance then shifts discontinuously from its resting value to a higher one which can also be induced by divalent cations, particularly Ba and Sr. The "response," therefore, is an augmented IR drop, not a shift in membrane electrode properties. The change evoked by threshold currents is local and its electrotonic spread has two components; the potential during the "response" is attenuated less, but is prolonged more than is the potential below threshold, since the space constant is increased in correlation with the higher membrane resistance. A similar anomalous effect occurs in electroplaques of *G. carapo* (Bennett and Grundfest, unpublished) and may be a phenomenon common among excitable membranes.

The effect of sulfhydryl compounds on the colloidal state of the cytoplasm of Arbacia eggs. HOWARD ROTHSTEIN.

Earlier work of Rapkine, Mazia and Heilbrunn has clearly indicated the importance of the SH–SS transformation for the colloidal changes that occur in the cytoplasm during the course of the mitotic process. An attempt has been made further to elucidate the relation between the SH–SS equilibrium and the sol-gel transformation.

Unfertilized eggs of *Arbacia punctulata* were exposed for 30 minutes to 0.075 *M* solutions of mercaptoethanol and 0.085 *M* solutions of thioglycolate. In both cases the viscosity of the protoplasm increased significantly.

As Mazia has shown, mercaptoethanol inhibits cleavage in the Dendraster and Strongylocentrotus eggs. This has now been shown also to be true for the Arbacia egg. In addition, it was demonstrated that this inhibition is due, at least in part, to a prevention of the reversal of the mitotic gelation. That is to say, the viscosity of the cytoplasm increases after 20 minutes (at 21° C.) just as it does normally, but it never decreases again as it does in the controls, and the eggs cannot divide.

SH inhibitors were also employed. Mercuric chloride (10^{-5} *M*), 10^{-4} *M* iodoacetamide and 10^{-3} *M* p-chlorophenylmercurisulfonic acid all inhibit cleavage, seemingly through a similar colloidal effect, *i.e.*, prolongation of the mitotic gelation. Wilson and Heilbrunn have shown this for p-chloromercuribenzoic acid as well.

O-iodosobenzoic acid (10^{-3} *M*) was also employed, but no effect could be detected, either on cleavage or the attendant viscosity alterations. Possibly this compound is reduced in a more or less non-specific manner within the cell. It should be noted also that these compounds do not affect the viscosity of the unfertilized egg.

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Studies by filter paper electrophoresis of the hormones controlling color changes in the shrimp Crangon. MURIEL I. SANDEEN AND MILTON FINGERMAN.

Extracts of circumesophageal connectives with the tritocerebral commissure attached of *Crangon* were applied, using hot air, to one-half inch wide Whatman No. 1 filter paper strips. Each strip was submitted to electrophoresis at 500 V. and 0.1–0.2 mA. for a given period of time using citrate-phosphate buffer at pH 5.2 or a borate buffer at either pH 7.8 or pH 9.0. Each strip was cut into desired sections which were washed for 30 minutes with sea water. Eyestalkless *Crangon* were used as assay animals for these sea-water extracts. At each pH used both the tail-darkening hormone and the body-lightening hormone were electropositive. Using pH 9.0 electrophoresis was carried out for 2, 3, 5 and 16 hours. In each case the greatest amount of tail-darkening hormone and some body-lightening hormone were recovered from the ¼-inch origin while the greatest amount of body-lightening hormone was recovered from the first inch toward the cathode. After 5 and 16 hours significant amounts of body-lightening hormone with little or no tail-darkening hormone were recovered from the second inch toward the cathode. No hormone was ever recovered beyond 3 inches from the origin, whether or not the extract was boiled prior to application to the paper. Similar experiments were carried out using extracts of the eyestalks submitted to electrophoresis for 5 hours. To detect the antagonistic tail-lightening activity one-eyed *Crangon* adapted to a black background were used as assay animals. In 4 out of 7 cases, regardless of the pH maximum, tail-lightening

activity was recovered from the first inch toward the cathode. In the other cases maximum activity was in the second inch.

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Intra-specific reaggregations in tunicates labeled with tritiated thymidine. SISTER FLORENCE MARIE SCOTT AND JOSEPH E. SCHUH, S.J.

Cultures of *Amaroecium constellatum*, in early stages of metamorphosis, were exposed to either 1 μC/l ml. or 5 μC/l ml. of tritiated thymidine in standing sea water for two hours. The cultures were refreshed in running sea water for three hours and then immersed in tritiated thymidine for another period of eight hours. Cultures from the same shedding were kept in running sea water for a corresponding period of time. Late in metamorphosis (12 to 18 hours), when the young adult organization is established, the following combinations were made: 1) two tritiated branchial baskets and one non-tritiated abdomen; 2) two non-tritiated branchial baskets and one tritiated abdomen. The three fragments were ejected from their tunics and inserted into the evacuated tunic of a two-week-old Amaroecium. They were macerated together in the host tunic, pressed into place by plugs of tunicin and kept in running sea water. At critical stages through five days of development, radioautographs were prepared from serial sections.

Two types of aggregates develop from the combinations in which an epidermal envelope is reconstituted to enclose the fragments: 1) single individuals resembling Siamese twins, with two branchial baskets variously joined to a common abdomen; 2) twins of unequal size; one the product of a fused branchial basket and abdomen, the other a regenerated zooid from the second branchial basket. In both types, each fragment reconstitutes itself into an integrated branchial basket or abdomen. From a cursory examination of the radioautographs, it is clear that the reconstituted members then unite to form functioning complex zooids in the cases of the Siamese twins, and typical zooids in the cases of the double fusions. The areas of junction between tritiated and non-tritiated fragments are easily traced in the radioautographs which provide a precise and diagnostic means of identifying and following tissues in their reconstituting activities and in their final reaggregation into organisms.

Paper chromatography of pericardial organs of crayfish. THOMAS SMYTH, JR.

Heartbeat in crustaceans is initiated by an intrinsic ganglionic pacemaker, modulated by extrinsic acceleratory and inhibitory nerves and by "neurosecretion" from pericardial organs lying outside the ostia. It has been reported that the pericardial secretion is mainly excitatory, but inhibition was found on occasion. In order to isolate the excitatory substance and possibly unmask an inhibitory one, paper chromatography of crayfish pericardial organs was tried. Only excitatory material was found.

This material is soluble in water and 50% ethanol, is not extracted by ether, withstands boiling and is non-volatile. Activity was lost by desalting with Permutit-50 and on storage of boiled aqueous extracts for two weeks in the deep freeze.

Hot water or hot 50% ethanol extracts of pericardial organs were spotted directly on washed Whatman # 1 filter paper. Extracts equivalent to the pericardial organs of two crayfish were used for each paper. Ascending methanol : water : pyridine (80 : 20 : 4) was the best solvent system tried. After drying, the papers were cut in strips, eluted with water, extracted with ether, lyophilized and redissolved in Tris-buffered van Harreveld's solution for assay on crayfish hearts. Activity resembling that of the crude extract was found only in strips corresponding to an R_f of 0.68. Since activity steadily disappeared, highest activity was recovered when the entire process was conducted without pauses between the various operations. Recovery was not improved by developing in a nitrogen atmosphere or by chromatogramming with added ascorbic acid. The active region was not obviously ultraviolet fluorescent or absorbing, did not react with Ehrlich's or Dragendorf's reagent, stained lightly with ninhydrin and with ammoniacal silver nitrate.

The excitatory substance is apparently not serotonin, as this had an R_f value of 0.20 (agreeing with Carlisle, 1956). Only a fraction of the activity was lost on boiling in normal HCl or on digestion with trypsin.

Relation of ultraviolet-ray dosage to modifications in fertilization and early development of Arbacia. CARL CASKEY SPEIDEL AND RALPH HOLT CHENEY.

Systematic studies were made of the modifications in fertilization and early development of the sea urchin induced by ultraviolet radiation. The experimental material (eggs, sperm, embryos) to be irradiated was exposed to ultraviolet rays of 2537 Å wave-length from a lamp 6 inches away. Exposures ranged from 2 seconds to 48 minutes. The principal studies were made on unfertilized eggs unilaterally exposed for periods of 0.25, 0.5, 1, 2, 4 and 8 minutes, respectively. These were fertilized with normal sperm and kept protected from light. By special techniques, samples from each series were examined and compared continuously for effects on fertilization membrane, movements of granules and other visible protoplasmic structures, delay in time for first and later cleavages, and irregularities in blastomere formation. Later observations at frequent intervals were sufficient to reveal the ultraviolet-correlated changes in both locomotion and structure that accompanied the successive periods of differentiation of blastulae, gastrulae, and plutei. Each experiment was based on a graded series of ultraviolet exposures, gametes from the same pair of sea urchins being used in each case. A definite injury series was established for both irradiation of unfertilized eggs (subsequently fertilized) and irradiation of fertilized eggs. A direct relationship was noted between dosage and 1) amount of delay in cleavage and later development, 2) percentages of abnormalities produced, and 3) maximal growth attained. Such relationship was readily observed for the more severe doses; it was firmly established also for very mild doses. It was significant that many of the abnormalities resulting from ultraviolet radiation resembled those noted in our parallel x-ray experiments. Illustrative cinephotomicrographs of normal and fast-motion types were obtained which give a vivid portrayal of ultraviolet irradiation effects.

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The extractable proteins of sea urchin embryos. MELVIN SPIEGEL AND EVELYN SCLUFER SPIEGEL.

A recent investigation by Kane and Hersh (1959) on the ultracentrifugation of extractable proteins of sea urchin eggs revealed that the bulk of the extractable material was found in two major components. These authors suggested that it was very likely that other soluble proteins were present in the extract but that each must be present in such small concentration that it did not contribute to the observable Schlieren pattern. It was also suggested that the two major peaks themselves are heterogeneous. It was felt that perhaps another method of analysis would reveal whether such heterogeneity exists. Accordingly, 0.1 *M* KCl extracts, pH 7.0, of unfertilized eggs of *Arbacia punctulata* were prepared, adhering as closely as possible to the method of Kane and Hersh. The extracts were then dialyzed in the cold against three changes of Veronal buffer, pH 8.6, $\mu = 0.05$, overnight. The dialysate was centrifuged at 56,000 g for fifteen minutes, the supernate removed by aspiration and subjected to electrophoresis in the Model 38 Perkin-Elmer Tiselius Apparatus at 4° C. and 4 ma. constant current. The results revealed four major components, plus one and possibly two minor components. These relatively simple extracts possessed strong antifertilizin activity when tested against egg jelly, indicating that this procedure may be useful as a starting point in the isolation of egg antifertilizin. Substitution of 0.03 *M* carbonate buffer, pH 10.7, as the extracting medium and using this buffer for electrophoresis at 4° C., 4 ma. constant current, demonstrated eight components. Extracts made of blastulae, gastrulae, and plutei had essentially identical electrophoretic patterns. Attempts are being made to further characterize these components and to relate them to the proteins characterized by ultracentrifugation.

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The magnitude of random threshold fluctuations in the squid giant axon. CHARLES F. STEVENS.

It is known that in the frog node threshold varies randomly around a mean value according to a Gaussian density function with a standard deviation of 1.5–2.0% of the mean, and that there

is a variation in latency for a constant stimulus corresponding to this "excitability" fluctuation. In view of these threshold variations in the frog node, it is of interest to investigate the threshold variability of the squid giant axon, in order to determine to what extent any deterministic model may be a fair approximation to the actual case.

A 50 mu axial platinum electrode was inserted into an uncleaned 2½-cm. length of the giant axon from the hindmost stellar nerve of *Loligo pealii*, and constant current pulses were delivered by a modified Grass S4 stimulator which permitted stimulus intensity to be varied reliably by amounts on the order of 1/1500. The sharpness of the threshold for the membrane action potential was determined directly, and also indirectly by measuring latency fluctuations for a constant stimulus. These methods give the sum of fluctuations due to threshold variability and inconstancy of the stimulus.

The principal results are: (1) The threshold determined directly is sharp as far as could be determined by changes of stimulus intensity of 1/1500. (2) There is a latency fluctuation which is largest near threshold, and is, at its maximum, on the order of 10–20 microseconds. (3) A latency variation of this magnitude corresponds to a threshold plus stimulus variation with a standard deviation of 0.015 to 0.020% of the mean.

It is concluded that any random threshold variations in the squid giant axon have a standard deviation less than or equal to 0.02% of the mean. This is considered sufficiently small to justify the application of deterministic models.

Chemically induced hypothermia in the rat: the effect of oxazolium salts compared with 2,4-Dichlorophenoxyacetic acid, ("2,4-D"). F. N. Sudak, C. Lloyd Claff, J. R. Blumstein and F. J. Teyan.

The effects of the oxazolium salt (3-methyl-2(1-naphthyl)-5-phenyloxazolium-*p*-toluenesulfonate) and 2,4-dichlorophenoxyacetic acid on body temperature regulation in male albino rats during cold exposure were compared. Skin and deep colonic temperatures were recorded and carbon dioxide production was measured during a 90-minute exposure to an ambient temperature of 2.0° ± 0.5° C. Saline injected animals, used as controls, showed an increase of 1.5° C. in core-to-skin thermal gradient within 30 minutes after exposure to cold. This thermal gradient was maintained for the next 60 minutes. Colonic temperatures remained unchanged during the entire experiment. In the animals treated with "2,4-D" (300 mg./kg.) and in those treated with oxazolium salt (100 mg./kg.), no significant change in core-to-skin thermal gradient occurred. Colonic temperatures declined in a linear manner at the rate of 0.18° C./min. in both experimental groups.

Carbon dioxide production increased significantly (200–225% above "basal") in the control group within 15 minutes and remained at this level for the remaining 75 minutes. No significant rise (above "basal") in metabolism occurred in the two experimental groups. CO_2 production remained unchanged for 45 minutes and then declined steadily to reach a level 35% below "basal" after 90 minutes in the group treated with the oxazolium compound. Carbon dioxide production declined after 15 minutes in the cold in animals treated with "2,4-D" and reached a level 57% below "basal" at 90 minutes.

Animals treated with the oxazolium salts recovered their normal body temperatures (from 21.0° to 38.0° C.) within 3 hours when placed in an isothermic environment. Recovery of normal body temperature was much slower in animals treated with "2,4-D." Four days after the experiment, 6 of 8 animals treated with the oxazolium salt were dead. No deaths occurred among the "2,4-D"-treated rats for as long as 6–8 weeks (experiment discontinued at this time.)

Muscular activity in albino rats treated with 2,4-Dichlorophenoxyacetic acid, ("2,4-D"). F. N. Sudak, C. Lloyd Claff, F. J. Teyan and J. R. Blumstein.

Muscle potentials of male albino rats treated with 2,4-Dichlorophenoxyacetic acid were studied during lowering of body temperatures under light anesthesia induced with sodium pentobarbital or chloralose. Carbon dioxide production was measured at 15-minute intervals. Rats injected with physiological saline were used as controls. Deep colonic temperatures of the control animals fell sharply during the first 15 minutes and reached a steady state (average of 30.2 ± 1.0° C.) after 60 minutes in an ambient temperature of 12.0 ± 0.5° C.

Recordings of electrical activity in muscles made at each 0.5° C. fall in colonic temperature showed that a sharp rise in activity occurred in the control group at colonic temperatures between 36° C. and 35° C. Muscle potentials continued to rise in amplitude and the frequency of bursts of activity increased as lowering of the body temperature progressed. Muscle potentials reached a maximum amplitude of up to 700 microvolts (average of 7–5 peak-to-peak measurements during high frequency bursts) during the steady-state period. An increase in CO_2 production accompanied the increase in muscle activity and remained constant after 60 minutes in the cold.

In the group of animals treated with "2,4-D," colonic temperatures fell steadily (0.16° C./min.) and a steady-state temperature was never reached during 90 minutes in the cold. CO_2 production did not change significantly for the first 15 minutes and then declined with body temperatures. Electrical activity was not present to any significant degree (122 microvolts recorded in only one animal) at any time in the muscles of animals in this group. The evidence presented indicates that one of the mechanisms of action of "2,4-D" on thermoregulation in the rat is the prevention of shivering.

Thermal gradients in rats treated with 2,4 Dichlorophenoxyacetic acid, ("2,4-D"). F. N. Sudak, C. Lloyd Claff, F. J. Teyan and J. R. Blumstein.

The mechanism of action of 2,4 Dichlorophenoxyacetic acid on physical thermoregulation in rats was investigated. Core-to-skin thermal gradients were measured with thermistors placed 7 cm. into the colon (to a level just caudal to the kidneys in an animal of 150 grams body weight) and on an area of depilated skin just above the first thermistor. Temperature measurements were made on animals in a thermo-neutral environment (28–30° C.) and during a 90-minute exposure to a temperature of 2.0 ± 0.5° C. The average temperature gradient between the core and skin of both control and "2,4-D" treated animals exposed to isothermic temperatures was 1.3 ± 0.2° C. A significant increase (1.3 ± 0.2–2.8 ± 0.4) in the core-to-skin thermal gradient occurred in the control group after 30 minutes in a cold environment and was maintained for the next 60 minutes. Colonic temperatures remained constant during the entire experiment. No significant change in thermal gradient occurred during the 90-minute exposure to cold in the groups of animals treated with "2,4-D." A concomitant decrease in both colonic and skin temperature occurred in a linear manner (0.18° C./min.) in these animals. The evidence presented indicates that 2,4 Dichlorophenoxyacetic acid interferes with those mechanisms which control thermal conductivity in the skin of rats. The possible sites of action of this compound are discussed.

Oxygen consumption in Uca pugnax and pugilator before and after eyestalk removal. F. J. Teyan, F. N. Sudak and C. Lloyd Claff.

Oxygen consumption was measured in individual fiddler crabs before and after eyestalk removal. The operations were performed at room temperature. Eyestalkless crabs were divided into two groups: Group I, eyestalks removed and the stubs cauterized with a cautery needle; Group II, eyestalks removed and the stubs left uncauterized. Crabs with their eyestalks intact served as controls. Oxygen consumption was measured every half hour over a four-hour period on four consecutive days, using a modified Scholander method. Oxygen consumption increased within 24 hours after eyestalk removal in the group which was left uncauterized. O_2 consumption either remained the same or decreased over the four-day period in the group in which the stubs of the eyestalks were cauterized. Evidence is presented which indicates that the changes in oxygen consumption in eyestalkless fiddler crabs may be due to the method used in the removal of this organ.

Induced fluorescence in marine eggs. Kenyon S. Tweedell.

Secondary fluorescence was induced in embryological stages of Arbacia, Chaetopterus, Aeolis and Pectinaria with fluorochromes and studied with ultraviolet light. A high pressure mercury vapor lamp (G. E.) with heat and exciter filters was coupled to a standard monocular microscope having a front surfaced mirror and barrier filter. Several vital fluorochromes,

acriflavine, thioflavine and acridine orange were used in concentrations ranging from .001% to .00001% in filtered sea water. Eggs were dyed immediately after shedding (or at various times post fertilization) for 3–5 minutes and then washed in fresh sea water to remove background fluorescence. In the lower concentrations, the eggs developed through the larval stage without being removed from the dye. In either case, the induced fluorescence is retained by the eggs throughout their development. The adaptability of such eggs for cytoplasmic studies is directly related to the amount of yolk platlets, pigment particles, etc. in the cytoplasm. Arbacia was thus unsuitable for study in the early cleavage stages. In other eggs, red, green or gold cytoplasmic granules were traced from early cleavage stages into the larvae. In Chaetopterus, the germinal vesicle and early nuclei appear as darker clear areas surrounded by fluorescing yellow-green yolk platlets. Aeolis fluoresces in various shades of green; thioflavine further differentiates the membrane. Eggs of Pectinaria fluoresce pale green with an orange granular periphery, light green nucleolus in the germinal vesicle, all enclosed in an orange membrane. In all species, spermatozoa appear bright green and their approach and contact with the egg are discernible. Polar body formation and the spindle area in successive cleavages are clearly defined. During the blastula, gastrula and larval stages, the nuclei fluoresce dark green. Fluorochromed embryos which die during development exhibit the Strugger effect; the cytoplasm changes to a brilliant orange and the nuclei fluoresce opaque green.

The effect of bacteria on radiophosphorus uptake of Fucus vesiculosus. WALTON D. B. WATT.

A study was made of the exchange of radiophosphorus between *Fucus vesiculosus* and sea water. The results are expressed as turnover time of plant tissue which is the time required for the total phosphorus in the tissue phase to pass into and out of that phase. Within the limits of the experiments (about 500 ml. of sea water, 2–5 gm. dry weight of *Fucus* and 1 μc. of P^{32}) turnover time was independent of biomass and volume of water. All experiments were conducted in darkness at 23° C. The amount of radiophosphorus (added as phosphate) in the water declined exponentially to an equilibrium value which remained constant for at least 60 hours, an indication that no measurable net uptake of phosphorus occurred during this period. The mean turnover time in freshly collected sea water was found to be 18.8 hours. When the antibiotic tetracycline hydrocloride was dissolved in the sea water (100 mg./l.) the mean turnover time became 37.6 hours. Each value is the mean of four experiments and the difference is highly significant ($p < 0.01$). When autoclaved or millipore-filtered sea water was used, values for turnover time showed close agreement with those obtained using antibiotic. The increase in turnover time with the reduction in bacterial populations was accompanied by an increase in the radiophosphorus accumulated in the algal tissue, suggesting that aquatic bacteria take up the dissolved inorganic phosphorus and release it again in some form (probably organic) which is not immediately available to the plant.

Effects of imposed electrostatic field on rate of locomotion in Ilyanassa. H. MARGUERITE WEBB, FRANK A. BROWN, JR. AND WILLIAM J. BRETT.

Animals were tested by determining the time required to travel a distance of 16 cm. along a section of a plastic tray located directly above a glass-encased 20×40 cm. copper plate. There was a similar copper plate 20 cm. above the test chamber. The copper plates were connected with a constant voltage power supply. In these experiments animals were paired, with one snail being run without electrostatic field for 5 trials and then in an electrostatic field for 5 trials. The other member of the pair was tested for 5 trials with an imposed electrostatic field and then for 5 trials without the field. Voltages used were 350, 500, and 1000. When all of the experiments conducted at 8, 9 and 10 A.M. are considered together it is found that the average test time is reduced significantly when an electrostatic field is imposed. The amount of reduction is 1.7% ($N = 224$, $p < 0.02$, > 0.01). When results obtained in the cases where the experimental situation prevailed in the first 5 trials are considered separately the mean rate is not significantly different from that of the controls. When the experimental condition prevailed in the second 5 trials the mean rate is 3% below the control value ($N = 95$,

$p < 0.001$). In afternoon tests the application of 500 and 1000 volts resulted in a 1.9% *increase* in time required for a trial ($N = 200$, $p < 0.001$). The application of 350 V resulted in a 3.5% *decrease* in time for a trial ($N = 50$, $p < 0.001$). Thus the effect of 350 V is in the same direction as in the morning and is of greater magnitude while the effect of 500 and 1000 V is in the opposite direction and of about the same magnitude.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Fluctuations in rate of locomotion in Ilyanassa. H. Marguerite Webb, Frank A. Brown, Jr. and William J. Brett.

The test chamber consisted of a plastic tray divided into 3×16 cm. sections. The tray was located directly above a glass-covered copper plate and 20 cm. below a similar copper plate. The test chamber was illuminated from one end by a lamp with a 60-watt bulb at a distance of 50 cm. For each test the time required for a snail to travel 16 cm. was determined. Snails which failed to cover the distance in 70 seconds in two preliminary trials were not used. Each animal was tested either 5 or 10 times depending on the experiment. Experiments were performed only between the hours of 8 A.M. and 8 P.M., E.S.T., from July 6 to August 14. For each hourly value 20 animals were timed for 5 trials each. A daily rhythm was observed in rate of locomotion with maximum rates occurring at 10 A.M. and 2–4 P.M., and minimal rates at 8 A.M., 12 noon, and 6 P.M. The amplitude of the fluctuations throughout the day represents 20% of the mean daily rate. A comparison of mean daily rates obtained at the same hours of different days showed fluctuations with an amplitude of 29% of the over-all mean daily rate. The rates throughout a series of tests on a single animal were found to vary systematically in a manner characteristic of the time of day. In the morning hours trials 2–5 averaged 1.5 sec. less than the first trial ($N = 100$, $p < .001$) and the reduction was maintained through runs 6 to 10. The mean of rates 2–5 in the afternoon showed a reduction not significantly different from that observed in the morning, but trials 6 to 10 yielded a mean rate not significantly different from 0. These variations in a single test series are of the order of 2 to 3%.

This study was aided by a contract between the Office of Naval Research, Department of Navy, and Northwestern University, NONR-122803.

Graded and all-or-none electrically excitable responses of lobster muscle fibers. Robert Werman and Harry Grundfest.

Intracellular depolarization produces only graded responses in stretcher and bender muscle fibers. These arise with vanishingly brief latency to strong stimuli and exhibit refractoriness. Long-lasting depolarizing pulses cause repetitive, damped responses and prolonged strong depolarization produces inactivation. Simultaneous recordings at several intracellular sites demonstrate that the responses propagate decrementally. The maximal amplitudes were 20 to 30 mv, appreciably smaller than the electrically excitable component elicited by stimulating the excitatory axon. This difference may be correlated with the wide distribution along the muscle fiber of excitatory depolarization by the multiterminal nerve supply. The membrane conductance increases about 25% during the response. Replacement of part or all the Na of the bathing fluid with Ba increases the membrane resistance 2- to 10-fold. Stimuli now evoke all-or-none responses of 50 to 100 mv, the amplitude tending to increase with Ba concentration. The responses are prolonged (durations up to 5 seconds), with development of a plateau after the peak. These responses also exhibit properties characteristic of electrical excitability: vanishingly brief latency, marked refractoriness, and inactivation by strong depolarization. Brief hyperpolarizing pulses applied during the plateau can terminate the response. At the peak of the spike, the conductance is increased about 400%, falling slowly during the plateau, and returning to the resting level only after the spike terminates. The impulses are propagated decrementlessly. However, simultaneous recordings at different sites in a muscle fiber reveal different configurations in the responses, which indicate spatial and temporal non-uniformities in the membrane. Unlike insect muscle fibers (Werman and Grundfest, *Fed. Proc., 1959, 18, 169*), Ba-treated lobster muscle fibers do not fire spontaneously.

Mechanism of Ba action on arthropod muscle fibers. ROBERT WERMAN AND HARRY GRUNDFEST.

The conversion of graded, electrically excitable responses of lobster muscle fibers to all-or-none spikes by Ba appears to be due to interference of the ion with K permeability. The resting membrane is increased 2- to 10-fold by low concentrations of Ba with hyperpolarization up to 15 mv. While addition of high potassium to the bathing medium causes depolarization and decreased membrane resistance, simultaneous addition of low concentrations of Ba leads to hyperpolarization and increased membrane resistance. Thus, Ba appears to inhibit K conductance and/or to render the membrane insensitive to K. Loss of membrane rectification, which also results, indicates reduced membrane sensitivity to unequal K distribution, probably caused by diminished K conductance. Since K conductance is probably responsible for repolorization of the action potential, its block by Ba can account for the high amplitudes of the responses, their all-or-none character, and prolonged durations after Ba. Replacement of K by Na, or NH_4 also causes hyperpolarization, although membrane resistance increases only up to 60%. However, all-or-none responses were never seen under these conditions.

In addition to its effect on K conductance, Ba can substitute to a limited extent for Na. After replacement of Na by choline has eliminated responsiveness, application of a 250 mM/L Ba solution frequently restores all-or-none responses. These spikes also are inactivated by depolarization, and, in this condition, brief hyperpolarizing pulses evoke spikes. Thus, the transport and inactivation mechanisms which normally utilize Na during their operation in electrically excitable responses can also utilize Ba. The similar effects of Ba on insect muscle fibers (McCann, Werman and Grundfest, *Biol. Bull., 1958, 115, 356;* Werman and Grundfest, *Fed. Proc., 1959, 18, 169*) suggest that the nature of graded responsiveness in these muscle fibers and the mechanism of Ba action are essentially similar to those in lobster fibers.

Action of x-rays on Paramecium: survival and reproduction. RALPH WICHTERMAN.

Pedigreed clonal cultures of *Paramecium calkinsi* from salt water of West Barnstable and *P. trichium, P. multimicronucleatum* and *P. bursaria* were investigated in regard to the biological effects of x-irradiation on survival and reproductive ability. All species were cultivated in standard lettuce medium with *Aerobacter aerogenes* as the food source. For *P. calkinsi,* the infusion consisted of two parts of lettuce medium and one part of filtered sea water. The irradiation chambers were sealed Nylon syringes of 2 ml. capacity. Usually 200 specimens were placed in each syringe and irradiated, free of air, in steps of 50,000 r up to 450,000 r. After a given dosage, 0.2 ml. of fluid containing a small number of specimens was expressed from a syringe into bacterized spot plates for observation and the establishment of *survival-reproduction* curves. Four syringes were irradiated at one time, each containing a different species. Data were obtained upon 307 isolations in which the fate of 2605 organisms was determined.

Instead of employing the conventional survival-curve in which dosage is plotted against per cent survival, a new type was devised for a minimal 48-hour period which appears to be more expressive of results. Called "survival-reproduction" curves, time in hours is plotted against not only survival of specimens for a given dosage (0–100%) but their subsequent reproductive rates as well. Among other things the data and curves show that *P. trichium* is not only the most radiosensitive but recovery and reproduction occur only at the lower dosages (survival-reproduction rate, 48 hours: control, 278%; 50 kr., 200%; 100 kr., 135%; 200 kr., 28%; 250–400 kr., 0%). On the other hand, *P. calkinsi* is the most radioresistant, showing relatively fast recovery and reproductive ability even after moderately high dosages (survival-reproduction rate, 48 hours: control, 468%; 200 kr., 308%; 250 kr., 267%; 300 kr., 115%; 400 kr., 70%; 450 kr., 18%).

In many instances within the species, the data and curves reveal that with moderately high dosages, reproduction is blocked temporarily for 24–30 hours after which there is not only recovery from irradiation effects but a gradually increased reproductive ability. The greater the dosage, the slower the recovery to reproductive ability.

In nearly all cases, those irradiated specimens which recover to yield large mass cultures have a reproductive rate comparable to the unirradiated controls.

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Responses of the skate, Raja, to hyperglycemic agents. PAUL A. WRIGHT.

The average normal blood sugar value for 20 freshly-caught specimens (probably of mixed species, *ocellata* and *erinacea*), as determined by the Nelson procedure during July and August, was 40.6 mg.% with a great range of variability from a low of 2 to a high of 120 mg.%. Epinephrine (Adrenalin chloride, Parke-Davis), injected intra-arterially at a dosage of 200 mcg./kg., induced a maximal increase in blood sugar of only 14 mg.%, reminiscent of the poor response of bullfrogs to this agent. Cortisone acetate, Merck (1 mg./kg.), did not raise the blood sugar level significantly. Glucose injected into the truncus in a dosage calculated to raise the blood sugar level by 100 mg.% (on the basis of 6% of body weight as the total blood volume) was converted at the following rates: 36% in 5 minutes, 49% in 30 minutes, and 85% in 60 minutes. On the basis of these determinations, the glucose tolerance curve for the skate has markedly different characteristics from that determined previously for the bullfrog. Nine of 10 animals responded to intra-arterial crystalline glucagon with a maximal hyperglycemic rise of 40 mg.% in 60 minutes at a dosage of 100 mcg./kg. and an increase of 68 mg.% in 80–90 minutes at a dosage of 200 mcg./kg. The skate's hyperglycemic reaction to glucagon, then, is comparable to that of the bullfrog but is unlike that of the angler fish (*Lophius piscatorius*), which seemed to be completely refractory to the hormone during the summer of 1958.

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LALOR FELLOWSHIP REPORTS

Functional modifications of sperm structure. MAURICE H. BERNSTEIN.

Electron microscopic investigation of the sperm of *Arbacia punctulata* reveals a wedge-shaped head 1 μ across at the base and 2 μ long. The anterior end has a cup-shaped depression which houses the acrosome granule. The posterior end is indented by the insertion of the tail. The mid-piece is composed of a single ring-shaped mitochondrion with a central hollow, itself doughnut-shaped, containing the centriole and the attachment structures of the tail. The tail has a pair of central filaments with nine paired filaments in radial array forming an outer ring. The central filaments are not present in the proximal half of the mid-piece, but the filaments of the outer ring continue and ultimately participate in the formation of the tail attachment.

Egg-water preparations are known to elicit acrosome reactions in sperm of many marine invertebrates. In egg-water *Arbacia* sperm exhibited a transitory agglutination reaction. Electron microscopy revealed the acrosome still intact, but the mitochondrion of the mid-piece expanded almost explosively.

In nicotin-induced polyspermy release of the acrosome granule to the egg surface was observed. An expansion of the mid-piece comparable to that seen in egg-water preparations was also found. Further direct observations on inseminated eggs have not been made.

In an attempt to reconstruct events following sperm entry, observations were made on sperm suspensions in sea water egg homogenates. An explosive membrane elevation of the sperm nucleus was produced. The tail and the mid-piece seemed very little affected.

The deoxyribonucleoprotein in sea urchin sperm can be solubilized in distilled water after preliminary treatment with a chelating agent and subsequent washing in distilled water. Electron microscopic observations on sperm in the early phases of extraction showed that the head membrane was absent or ruptured, and the nucleoprotein of the head was emerging into the medium.

These and similar experiments aimed at elucidating the modifications of sperm structure and their relations to fertilization are being continued.

Respiration and motility in Spisula spermatozoa. PIERRE H. GONSE.

Experiments have been performed in order to study oxidative metabolism and relate rates of oxygen consumption with motility. Spermatozoa were collected from the punctured gonad. Results described here refer only to material obtained from ripe animals. The spermatozoa

were suspended and washed once (by centrifugation at 3000 RPM) in sea water buffered by 0.02 *M* glycylglycine at pH 7.5. Final cell density was from 10 to 30×10^8 cells/ml. Oxygen consumption was measured by polarography; motility estimated by microscopic examination. For endogenous respiration Z O_2 values of 1 were obtained.

"Difference spectra" have been recorded at room or liquid nitrogen temperature in the laboratory of Dr. Britton Chance. In anaerobic suspensions of spermatozoa after treatment by 1 mM KCN, one observes absorption bands corresponding to reduced cytochromes *a*, a_3, *c*, and in the region of the *b* cytochromes, bands at 557 and 562 mμ (low temperature). In the presence of carbon monoxide a cytochrome a_3–CO complex is formed.

The endogenous respiration was augmented 6 to 9 times by 5×10^{-5} *M* dinitrophenol (DNP), a concentration which stops motility completely. A 60% inhibition of respiration occurs in the presence of 5 mM amytal, motility being only slightly lowered. After DNP activation respiration is more sensitive to amytal; a 5 mM concentration produces 90% inhibition. Respiration is unsensitive to 20 mM malonate.

Titrations with azide show two distinct phenomena. The endogenous respiration is stimulated up to 2 times at azide concentrations of 1 to 3 mM, and in this range motility is completely arrested. At higher concentrations this activation and an inhibition are superimposed; different samples yield various results depending upon the degree of activation by 1–3 mM azide. When activation is low, 60% of the respiration is inhibited by 10 mM azide. In the presence of DNP the respiration, which is 8 times greater, is more sensitive to azide; inhibition starts with 0.05 mM azide and is 95% at 10 mM azide. With or without added DNP, the titrations end with the same absolute value of oxygen consumption resistant to azide 10 mM. The inhibition exerted at the level of cytochrome oxidase is therefore a function of the respiratory rate. The activation of respiration at lower azide concentrations may be due to an uncoupling action as in bull spermatozoa.

In *Spisula* spermatozoa motility is more sensitive to uncoupling than to a lowering of the oxygen consumption.

The influence of ribonuclease on the development of the sea urchin. VINCENZO LEONE.

In order to study the proteic organization of sea urchin embryos I have performed experiments that would make understandable the meaning and the importance of ribonucleic acid for morphogenesis of the embryo. I studied the effects of the treatment with ribonuclease on *Arbacia punctulata* (Lam.) embryos, from the unfertilized egg to the early mesenchyme blastula stage. The experiments have been carried out in the following way:

1) Unfertilized eggs were placed in the RNase solution (1‰ in sea water crystalline RNase Sigma) for three hours, and then fertilized; 32% of the embryos reached the pluteus stage, 34% failed to develop entoderm and 34% died.

2) Eggs, immediately after fertilization, were placed in the RNase solution, where they remained for three hours. Twenty per cent of the embryos reached the pluteus stage, 40% failed to develop entoderm and 40% died.

3) A three-hour treatment with RNase was performed on embryos at young blastula stage (that is, 3 to 6 hours after fertilization). The result was: 60% of the embryos reached the pluteus stage, 10% failed to develop entoderm and 30% died.

4) After a 3-hour RNase treatment on swimming blastulae (from the sixth to the ninth hour after the fertilization) 50% of the embryos reached the stage of plutei, 20% failed to develop entoderm and 30% died.

5) Finally, the treatment on early mesenchyme blastula stage (from the ninth to the twelfth hour after fertilization) produced the following result: 50% reached the pluteus stage, 17% failed to develop entoderm and 33% died.

RNase acts by disturbing, in some way, the developmental processes and especially the formation of entoderm. It does not show an animalizing effect; in fact, the ciliary tuft is not increased and the mesenchyme is formed, sometimes in excess. Working with an enzyme that disturbs the embryonic development but does not exhibit animalizing or vegetalizing effect evoked interest in testing the reduction gradients of the embryos by treating them with Janus green 1 : 200,000. As control I used normal and trypsin-animalized embryos and also SCN-

and iodosobenzoic acid-animalized embryos. The reduction, as it is known, starts earlier in the animalized than in the normal embryos. The reduction activity of the RNase-treated embryos was found to be lower than that of the animalized series and comparable with that of normal embryos.

A rapid method for measuring total lipid, applied to the analysis of the lipids and lipoproteins of Arbacia eggs and embryos. JULIAN B. MARSH.

Lipids were extracted by the method of Folch, Lees and Sloane Stanley which employs 19 volumes of 2:1 chloroform-methanol followed by mixing with 0.2 volume of water and centrifugation. The washed chloroform extract was taken to dryness in test tubes and heated to boiling with 1 ml. of concentrated H_2SO_4 over an open flame. After cooling, 9 ml. of water were added rapidly and the turbidity measured in a Klett photometer with a green filter. A standard curve with palmitic acid was linear between 0.2 and 1.2 mg. In 34 duplicate analyses, the average error was 3.9% with a standard deviation of 5.3%. Expressed as mg. per mg. of protein, jelly-free Arbacia eggs contained the following: total lipid, 0.50; phospholipid, 0.14; unesterified sterol, 0.041; total sterol, 0.046. No significant changes in these ratios were found 5 minutes after fertilization or after 1, 5, 8, and 22 hours of development. After centrifugation of *M* sucrose homogenates of eggs, the mitochondria were found to contain 38% of the total lipid, 75% of the phospholipid and 44% of the sterol; the microsomes contained 5, 12 and 5% of these fractions, respectively; and the supernatant, 53, 7 and 47%, respectively. By preparative ultracentrifugation of the supernatant of an isotonic KCl homogenate of the eggs, a water-soluble lipoprotein mixture was obtained, having an S_f value of 38.5 at density 1.10. The mixture contained 25% protein, 10% phospholipid, 0.25% sterol and 65% triglyceride. It was separable into 5 components by electrophoresis in 0.1 *M* barbital buffer at pH 8.8.

Inorganic pyrophosphatase and the motility of spermatozoa. LEONARD NELSON.

Marine invertebrate and rat sperm contain an inorganic pyrophosphatase distinct from the flagellar adenosinetriphosphatase (ATP-ase). While Mg activates the ATP-ase more than does Ca, the IPP-ase exhibits an "absolute" dependence on Mg; Ca, Mn and Zn permit only negligible activity. Mn in the incubation medium in equimolar concentration with Mg (10^{-3} *M*) causes 75% depression of Mytilus sperm-tail IPP-ase. The relative concentrations of Na-pyrophosphate (NaPP) and Mg may influence, among other parameters, the pH-activity curve, which is bimodal with peaks at neutrality and in the alkaline range. The sperm tails also dephosphorylate Na-tripolyphosphate (NaTPP) and Na-hexametaphosphate (NaHMP) which, together with NaPP, comprise members of a series of polyphosphates. Increasing the concentrations of NaPP and NaTPP above their respective optima causes a sharp drop in enzyme activity. This may not be attributable to an inhibitory effect of accumulated reaction product since the enzyme activity against NaHMP levels off at a maximum in spite of the high inorganic phosphate content of the medium.

Electron micrographs of frozen-dried rat epididymal sperm show increased density of the nine outer longitudinal fibers after incubation in NaPP. After prolonged treatment with 50% glycerol, the fibers lose their reactivity with NaPP, although retaining a positive cytochemical reaction toward ATP. Since pyrophosphate serves as a plasticizer of glycerinated sperm (as well as muscle), these, together with other observations, suggest that control of sperm flagellation may depend on a contraction-relaxation cycle, mediated by the action of NaPP-IPP-ase system regulating the relaxation phase with ATP-ATP-ase dominating the contraction phase, possibly through their mutual requirement for Mg.

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Histochemical studies of the reproductive cycle of Ephelota coronata. EDWARD E. PALINCSAR.

The marine suctorian *Ephelota coronata* was used to study factors involved in abnormal reproduction and ageing in a unicellular system. Massive populations were found on *Tubularia* growing along the Woods Hole shore.

The following culture method increased the survival time in the laboratory from 48 hours to 10 days, but with a decreasing reproductive potential. *Campanularia* were inoculated with *Ephelota* and placed in standing sea water which was aerated continuously and filtered through activated carbon. Cultures were maintained at 20° C. at pH 8.0. *Campanularia* were fed daily on *Artemia*, and *Ephelota* were fed two *Uronema* per suctorian per day.

Preliminary histochemical investigations comparing young and adult suctorians were made. The scarlet R method for lipids indicated a 2.4% greater concentration in the adult as compared to young. On feeding there is a great increase in lipid content and the suctorians became opaque. Brachet's method for RNA and DNA showed little difference. Bourne's method showed low xanthine oxidase activity but no relationship to the age of the individual. The xanthydrol technique failed to detect urea. Alizarin Red S method revealed a 3.8% increase of calcium in aged individuals. The Schultz-Smith method and the Gomori method for uric acid gave positive results, but no concentration difference with age. A pigment, tentatively identified as a carotenoid, increased 62.4% with age. Using the mercuric bromphenol technique, a great amount of protein was found in the stalk. Preliminary studies indicate that the rate of synthesis or conversion of the stalk protein is related to the age of the parent individual. Lags of 15–40 minutes were noted in the aged. Preliminary evidence also indicates that the number of buds produced decreases by 20–50% in aged individuals and that the fewer the buds, the larger the total volume of the bud.

Effects of some purine analogs on cleavage in Arbacia punctulata. EDWARD E. PALINCSAR.

Effects of 8-azaguanine and pyrazoloisoguanine on early cleavage of *Arbacia* were examined in order to gain some insight into their action mechanism. The 8-azaguanine was used as the sodium salt. Fertilized eggs were placed in concentrations of the analog in sea water ranging from 5×10^{-5} *M* to 3×10^{-4} *M*. Temperatures were experimentally varied from 20–30° C. At 1×10^{-4} *M*, first cleavage was delayed 30–60 minutes, depending on temperature. Higher concentrations of 8-azaguanine were not used, since it crystallized in sea water. Thirty-minute pretreatments of unfertilized eggs yielded a 20-minute delay in the first cleavage. The effect of 10^{-4} *M* 8-azaguanine on the clear half, the red half and the clear quarter was determined, showing a 20-minute delay in the clear halves and a 15-minute delay in the clear quarters. Five- and 10-minute exposures of zygotes to 10^{-4} *M* 8-azaguanine during the course of first cleavage indicate that greatest inhibition occurs up to late prophase. Ten-minute treatment of unfertilized eggs in 10^{-4} *M* 8-azaguanine showed no change in viscosity, but did show an increase in cortical fluidity.

Using standard methods for the preparation of homogenates from ½ cc. of packed cells, an attempt was made, using spectrophotometric methods and Thunberg techniques, to determine xanthine oxidase activity during early cleavage and in the unfertilized egg. No correlation with 8-azaguanine was obtained and in fact, little to no xanthine oxidase was detected. The techniques may not have been sufficiently sensitive. Pyrazoloisoguanine, both as the hydrochloride salt (1×10^{-4} *M* in sea water), and as a saturated sea water solution, had no effect on cleavage, possibly due to a permeability barrier. These preliminary results suggest that 8-azaguanine action is related to the mitochondrial content of the cell, the period of active protein and nuclei acid synthesis during cleavage and to changes in fluidity of the cortical layer.

On the stability of the structures of animalized and vegetalized sea urchin embryos. SILVIO RANZI.

In previous research, I reached the conclusion that the solution of the proteins of animalized embryos are less stable than the solutions of the proteins of normal embryos, and that the proteins of vegetalized embryos are more stable than the proteins of the normal embryos. The object of the present research was to test this point on the living embryos.

Embryos of *Arbacia punctulata* (Lam.) were animalized by adding 1 ml. 0.5 *M* NaSCN to 9 ml. of egg suspension in sea water, before or after the fertilization. Animalization was also obtained, after fertilization, by 1.3×10^{-3} o-iodosobenzoic acid (IBA) in sea water.

Embryos were vegetalized by adding 1 ml. of 0.5 *M* LiCl to 9 ml. of egg suspension after fertilization. The period of treatment with all these substances was 6 hours. The animalized embryos developed about fifty per cent to plutei and fifty per cent to ciliated blastulae with very long and large ciliary tuft; vegetalized embryos developed about fifty per cent to exogastrulae and fifty per cent to ovoid larvae with a very large intestine.

On these embryos two different series of experiments were carried out.

In the first 2.5 ml. of urea 60 per cent were added to 7.5 ml. of the culture after careful washing in order to remove the previous acting substance. Instead of the urea, 0.2 per cent of trypsin in distilled water can be used (5 ml. trypsin solution to 5 ml. egg suspension). After 15–180 minutes the cultures were washed and put in pure sea water In all these experiments the mortality was very high, the maximum being found in the SCN- or IBA-treated cultures and the minimum in the Li-treated embryos.

The other series of experiments shows that IBA or other animalizing substances, added three hours after the fertilization to a culture of Li-treated embryos, decrease the percentage of vegetalized embryos.

The two kinds of described experiments show that it is possible to observe in the living embryo that the action of lithium is a stabilizing action on the protein structures that opposes demolition by urea, by proteolytic enzymes or by other animalizing substances. On the contrary, the action of animalizing substances lowers resistance to the urea and to proteolytic enzymes.

Further observations relating radiation-induced mitotic delay to centriole damage. RONALD C. RUSTAD.

Two new lines of evidence supporting the hypothesis that radiation-induced mitotic delay arises from damage either to the centriole or a nuclear "trigger" for centriole replication have been obtained.

In *Arbacia* a strong parallelism exists between the pre-fertilization recoveries of x-irradiated eggs from mitotic delay and multipolar spindle induction. Individual eggs dividing multipley after the recovery period are greatly delayed.

Earlier studies of cyclical changes in sensitivity to x-ray-induced mitotic delay were confirmed. Furthermore, the induction of multipolar spindles was found to be dependent on the mitotic stage in the same manner as mitotic delay sensitivity.

Parenthetical to the central problem, numerous observations were conducted on high radiation dose phenomena seen previously in *Lytechinus variegatus*. Tight membranes are sometimes formed. Hemispherical pockets 10 to 30 microns in diameter can appear between the hyaline layer and the cell surface two hours after fertilization. Migration of pigment granules into the furrow region is often enhanced. Micromere formation can be dissociated from the normal cleavage schedule, but not its own "clock," resulting in two- to eight-cell stages containing micromeres. Healthy half-blastulae with degenerating half-morulae appear.

Since the induction of multipolar spindles is accompanied by a retardation of aster multiplication rate and cells with odd-integer numbers of asters occur, the present experiments strongly suggest some form of centriole damage.

This work was performed under the tenure of a Lalor Fellowship.

The inhibition of mitosis in the sea urchin egg by acridine orange. RONALD C. RUSTAD.

Since the diaminoacridines exhibit differential fluorescence when attached to either RNA or DNA *in vivo*, experiments concerning the possible inhibition of mitosis by interference with nucleic acid metabolism in *Arbacia* eggs have been initiated.

Acridine orange (10^{-4} to 10^{-7} *M*) can block or delay mitosis when administered within 20 to 25 minutes after fertilization. Later addition affects the second rather than the first mitosis.

The dye is actively accumulated by the egg. The higher concentrations stain the jelly coat; removal of the jelly coat does not prevent mitotic inhibition. Condensation of part of the echinochrome within the pigment granules was noted at 10^{-4} to 10^{-5} *M* concentrations.

Sperm exhibit a time- and concentration-dependent loss of motility and ability to fertilize. Mitosis is delayed when eggs are inseminated with treated sperm. This sperm-induced delay in our present state of knowledge suggests an action on either the nucleus or the centriole.

If the highly selective delay pattern during the division cycle results from action on the same system that causes sperm damage, it would be reasonable to postulate interference with the nucleic acids of the nucleus or the RNA of the centriole. However, the possibility that the cyclical changes might arise from some other effect will require further investigation.

The sensitivity of sperm and the cyclical sensitivity changes of the fertilized egg to acridine orange treatment are very similar to the radiation-induced mitotic delay patterns. Various authors have attributed radiation delay to nuclear damage and the present author has suggested that the radiation effect arises from centriole damage.

This work was performed under the tenure of a Lalor Fellowship.

Neuromuscular mechanisms of sound production in Opsanus tau. C. R. SKOGLUND.

The functional activity of the intrinsic striated muscles of the swimbladder, which are engaged in sound production of the toadfish (Tower, 1906; Fish, 1954), has been studied by simultaneous recordings of action potentials, mechanical effects, and sound. Most experiments have been performed on the excised bladder kept in air at room temperature (21° C.).

The motor nerve was found to conduct at above 25 m/sec. The shape of the compound action potential indicated fairly uniform fiber sizes, which was confirmed by preliminary histological analysis (fiber diameter 10 μ).

The muscle consists of short fibers running transversely to the long axis of the muscle. A single maximal nerve volley caused a well synchronized contraction as verified by simultaneous recording from different points by steel microelectrodes.

When the muscle contraction was recorded by an RCA transducer with stylus placed against the muscle surface, the earliest detectable movement occurred during the falling phase of the muscle action potential. Contraction peak was reached within 5–8 msec, and relaxation was complete in an additional 5–7 msec. The changes in bladder pressure during contraction, measured by a capacitance manometer, showed a similar time course.

The sound recording with an electrodynamic microphone (60–10,000 cycles) was limited for technical reasons to the initial high-amplitude change. This was recorded as a di- or triphasic wave lasting about 2.5 msec, and audible as a "pop" sound. The first deflection was synchronous with the falling phase of the muscle action potential, and thus, the second occurred during the early phase of recorded muscle movement.

The data are used to interpret the natural sound production of the toadfish, of which some comparative studies have also been made; the results may be relevant to the general problem of muscle sound generation.